PC-SPES down-regulates COX-2 via inhibition of NF-κB and C/EBPß in non-small cell lung cancer cells

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Abstract. Aberrant expression of COX-2 occurs in many types of malignancies including colon and lung cancers, and is implicated in development and progression of cancer. The molecular mechanisms associated with aberrant expression of COX-2 in lung cancer cells remain to be fully elucidated. In this study, we found that non-small cell lung cancer (NSCLC) NCI-H520 and NCI-H460 cells constitutively expressed COX-2 and produced prostaglandin E_2 (PGE_2) as measured by Western blotting and enzyme-linked immunosorbent assay (ELISA), respectively. Reporter assays showed that transcriptional regulation of COX-2 was blunted when either the NF-IL6 (C/EBPß) or nuclear factor-κB (NF-κB) binding site in the COX-2 promoter was mutated, suggesting that C/EBPß and NF-κB transcription factors have an important role in aberrant expression of COX-2 in these lung cancer cells. In addition, the eight herbal mixture PC-SPES (Lot. 5431219) caused growth arrest and apoptosis of NCI-H520 and NCI-H460 cells in association with blockade of NF-κB and down-regulation of C/EBPß, resulting in down-regulation of COX-2 and PGE_2 in these cells. On the other hand, PC-SPES up-regulated the level of C/EBPß in these cells. Taken together, C/EBPß and NF-κB may be promising molecular targets for COX-2 inhibition in lung cancer cells. PC-SPES might be useful in the adjuvant setting for the treatment of individuals with resected NSCLC as well as other types of cancer in which COX-2 is activated.

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Abbreviations: COX, cyclooxygenase; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin; NF-IL6, nuclear factor for interleukin 6; NF-κB, nuclear factor-κB; AP-1, activator protein-1; C/EBP, CCAAT/enhancer-binding protein; ELISA, enzyme-linked immunosorbent assay

Key words: PC-SPES, COX-2, NF-κB, C/EBPs

Introduction

Cyclooxygenase (COX) is the rate-limiting enzyme for the production of prostaglandins and thromboxanes from free arachidonic acid (1,2). Two forms of COX have been described; a constitutively expressed enzyme, COX-1 and an inducible enzyme, COX-2. COX-1 is ubiquitously expressed. COX-2 is an inducible protein which is an essential component of the inflammatory response, as well as involved in the repair of injury (3). Physiological activity of this enzyme provides a benefit to the organism; however, the aberrant or excessive expression of COX-2 has been implicated in a wide variety of pathologic processes including arthritis, carcinoma, as well as septic shock. Abnormal levels of this enzyme have been observed in many types of cancer including those from colon and lung; and studies implicate its involvement in development and progression of cancer (4-6). However, the molecular mechanisms of aberrant expression of COX-2 in cancer cells remain to be fully elucidated.

PC-SPES contains a partially extracted mixture of eight herbs: *Dendrantherma morifolium*, Tzvel; *Ganoderma Lucidium*, Karst; *Glycyrrhiza glabra L*; *Isatis indigotica,* Fort; *Panax pseudo-ginseng*, Wall; *Rabdosia rubescens*; *Scutellaria baicalensis*, Georgi and *Serenoa repens* (7). In previous studies, we and others have shown that PC-SPES mediated an antiproliferative effect on prostate cancer cells *in vivo* and *in vitro* (8,9). Recently, we showed that PC-SPES inhibited the proliferation of colon cancer cells and prevented polyp formation in a murine colon cancer model, the Apc^{min} mouse (10). These mice have a germ-line, non-sense mutation at codon 716 of the adenomatous polyposis coli (*APC*) gene; and they spontaneously develop multiple polyps in their small and large intestines at the age of 10-12 weeks (11). Therefore, Apc^{min} mice are considered to be useful for analysis or prevention of human familial adenomatous polyposis (FAP) and sporadic colorectal cancers. The polyp formation was dramatically prevented in Apc^{min} mice when they were either treated with known COX-2 inhibitors (11,12), or the mice were engineered to have a deletion of the *COX-2* gene (11). These results indicated that PC-SPES might act as a COX-2 inhibitor.

In this study, to understand better the molecular mechanisms of aberrant expression of COX-2 in NSCLC

cells, we performed reporter gene assays using a series of mutated COX-2 promoters. Our data indicated that NF-IL6 and NF-κB binding sites of the COX-2 promoter played an important role in the aberrant expression of COX-2 in these cancer cells. Of note, the eight herbal mixture PC-SPES induced the growth arrest and apoptosis of NCI-H520 and NCI-H460 cells. In addition, PC-SPES down-regulated COX-2 transcripts and protein in conjunction with down-regulation of C/EBPß protein and inhibition of NF-κB activity.

Materials and methods

Cell line. NCI-H520, NCI-H460, and NCI-H1299 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and were grown in RPMI medium (Gibco Life Technologies, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (FCS, Gibco). Murine macrophage RAW264.7 cells were obtained from ATCC and grown in Dulbecco's minimal essential medium (Gibco) with 10% heat-inactivated fetal bovine serum (FCS, Gibco) containing no detectable LPS (<0.006 ng/ml as determined by the manufacturer).

Chemicals. PC-SPES (Lot. 5431219) was obtained from Botanic Lab, Inc. (Brea, CA). One capsule contains 320 mg of powdered herbal extracts. Stock solutions of PC-SPES were prepared by exposing these herbal extracts to 70% ethanol (one capsule/1 ml of 70% ethanol). The final concentration of ethanol was <0.02%, and all experimental conditions were compared with vehicle controls. Warfarin and indomethacin were purchased from Sigma Chemical Co. (St. Louis, MO). Oridonin was purified from PC-SPES as previously described (13).

MTT assays. Cells ($5x10^4$/ml) were cultured in the presence of various concentrations of PC-SPES (0.25-4.0 μl/ml) for 4 days in 96-well plates (Flow Laboratories, Irvine, CA). After culture, cell number and viability were evaluated by measuring the mitochondrial-dependent conversion of the tetrazolium salt, MTT (Sigma), to a colored formazan product. MTT (0.5 mg/ml in PBS) was added to each well and incubated for 4 h at 37˚C. The medium was carefully aspirated, and dimethyl sulfoxide (DMSO; Burdick & Jackson, Muskegon, MI) was added to solubilize the colored formazan product. Absorbance was read at 540 nm on a scanning multiwell spectrophotometer (Bio-Rad) after agitating the plates for 5 min on a shaker.

Assessment of apoptosis. Apoptotic cell death was examined by terminal deoxyribonucleotide transferase-mediated dUTP nick end labeling (TUNEL) method using the *In Situ* Cell Death Detection kit (Roche Molecular Biochemicals, Germany) according to manufacturer's instruction. For quantification, three different fields were counted under the microscope and at least 300 cells were enumerated in each field. All experiments were performed twice.

Transfections and luciferase assay. Wild-type and a series of mutant COX-2 promoters cloned upstream of the luciferase gene in the pXP2 vector were a generous gift from Harvey R. Herschman (University of California, Los Angeles) (14). Cells (10^4/ml) were plated on 24-well plates and on the following day, cells were transfected with the indicated plasmids using the GenePORTER transfection reagent (Gene Therapy Systems, Inc., San Diego, CA). Luciferase activity in cell lysates was measured by Dual Luciferase assay system (Promega, Madison, WI) and this was normalized by Renilla activities. All transfection experiments were carried out in triplicate wells and repeated separately at least three times.

Western blot analyses. To prepare whole cell lysates, cells were suspended in lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 100 μg/ml phenylmethysulfonyl fluoride, 2 μg/ml aprotinin, 1 μg/ml pepstatin, and 10 μg/ml leupeptin], and placed on ice for 30 min. After centrifugation at 15,000 x g for 20 min at 4˚C, the supernatant was collected.

To prepare nuclear extracts, cells were suspended in ice-cold extraction buffer containing 20 mM HEPES (pH 7.9), 20% glycerol, 10 mM NaCl, 0.2 M EDTA (pH 8.0), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 1 mM dithiothreitol, 100 μg/ml phenylmethysulfonyl fluoride, 2 μg/ml aprotinin, 1 μg/ml pepstatin, and 10 μg/ml leupeptin. After 10 min of incubation on ice, nuclei were collected by a short spin in a microcentrifuge. The supernatant was removed, and the nuclei were resuspended in ice-cold extraction buffer. After 30 min of incubation, supernatant was collected by centrifugation at 15,000 x g for 20 min at 4˚C. Protein concentrations were quantitated using a Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA). Proteins were resolved by 4-15% SDS polyacrylamide gel, transferred to an immobilon polyvinylidene difuride membrane (Amersham Corp., Arlington Heights, IL), and probed sequentially with antibodies: anti-p53 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-p21 (Santa Cruz Biotechnology), anti-C/EBP-α, -ß, anti-COX-1, -2 (Santa Cruz Biotechnology), and anti-actin antibody (Santa Cruz Biotechnology). The blots were developed using the enhanced chemiluminescence kit (Amersham Corp.).

Evaluation of NF-κB activity by enzyme-linked immunosorbent assay (ELISA). The DNA binding activity of NF-κB in lung cancer cells was quantified by enzyme-linked immunosorbent assay (ELISA) using the Trans-AM NF-κB p65 transcription factor assay kit (Active Motif North America, Carlsbad, CA), according to the instructions of the manufacturer. Briefly, nuclear extracts were prepared as previously described and incubated in 96-well plates coated with immobilized oligonucleotide (5'-AGTTGAGGGGACTTT CCCAGGC-3') containing a consensus (5'-GGGACTTTCC-3') binding site for the p65 subunit of NF-κB. NF-κB binding to the target oligonucleotide was detected by incubation with primary antibody specific for the activated form of p65 (Active Motif North America), visualized by anti-IgG horseradish peroxidase conjugate and developing solution, and quantified at 450 nm with a reference wavelength of 655 nm. Background binding was subtracted from the value obtained for binding to the consensus DNA sequence.

Measurement of prostaglandin E_2. NCI-H520 cells ($2x10^5$/ml) were plated in 6-wells and cultured with either PC-SPES (2 or 4 μl/ml) or control diluent. After 48 h, culture medium

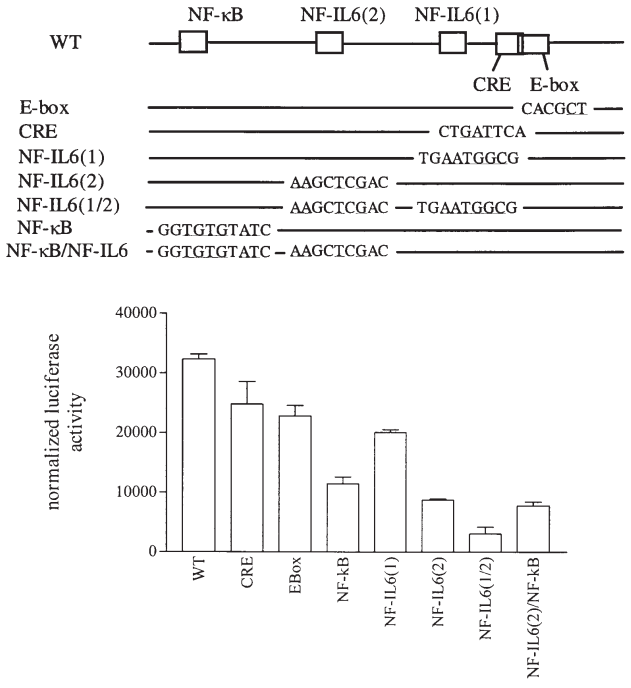

Figure 1. COX-2 promoter activity in NCI-H520 cells. The wild-type (WT) and a series of mutated COX-2 reporter constructs are shown at the top. NCI-H520 cells were transfected with the indicated reporter constructs (0.5 μg); and after 24 h, luciferase reporter activity was measured by a dual reporter assay system. pRL-SV40-luciferase (Renilla luciferase) vector was cotransfected for normalization. Results represent the mean ± SD of triplicate plates. This experiment was repeated independently three times, and similar results were obtained. Underlined nucleotides identify base changes from the wild-type sequence.

was collected and the concentration of PGE_2 was measured by ELISA kit (PharMingen, San Diego, CA).

Statistical analysis. Statistical differences were determined by two-tailed Student's t-test.

Results

Molecular mechanisms of constitutive expression of COX-2 in NCI-H520 cells. Lung cancer cells can express COX-2 (14). For example, we found constitutive expression of COX-2 in NCI-H520 and -H460 cells (Fig. 2A). To begin to investigate how COX-2 transcripts are constitutively regulated in lung cancer cells, NCI-H520 cells were transiently transfected with either the wild-type COX-2 promoter spanning nucleotides -724 to +7 cloned into firefly luciferase reporter plasmid pXP2 or the empty pXP2 plasmid (15). After 24 h, cells were harvested and reporter activity was measured. The cells transiently transfected with wild-type COX-2 promoter, increased their reporter activity about 600-fold as compared with the cells transfected with empty plasmid (data not shown). Mutation of either the 3' NF-IL6 site [NF-IL6(1)] or the 5' NF-IL6 site [NF-IL6(2)] in the COX-2 promoter decreased reporter gene activity by either 40% or 75%, respectively as compared with the wild-type COX-2 promoter (Fig. 1). When both sites were mutated, about 90% reduction of reporter activity occurred. Similarly, when the NF-κB site was mutated, reporter activity was reduced by 60% and combined mutation of the NF-κB and 5' NF-IL6(2) sites caused approximately 70% reduction. These data indicated that the transcription factors NF-κB and C/EBPß which bind to NF-κB and NF-IL6 DNA binding site, respectively played an important role in aberrant expression of COX-2 in NCI-H520 NSCLC cells.

Effect of PC-SPES on the expression of COX-2 and production of PGE_2 by NSCLC cells. Recently, we showed that the eight-herbal mixture PC-SPES inhibited the growth of colon cancer cells *in vitro* and prevented polyp formation in a murine colon cancer model, the APC^{min} mouse in which codon 716 of the *APC* gene was mutated (10). These results prompted us to hypothesize that PS-SPES could act as a COX-2 inhibitor. Therefore, the effect of PC-SPES on COX-2 was studied at the protein level. Western blot analyses showed that both NCI-H520 and NCI-H460 cells constitutively expressed COX-2 protein; and exposure of these cells to PC-SPES (4 μl/ml, 24 h) down-regulated the level of COX-2 by 50% and 60%, respectively (Fig. 2A).

As an additional evaluation of the effect of PC-SPES on COX-2 activity, we measured the level of PGE_2 in culture medium of NCI-H520 cells treated with PC-SPES (Fig. 2B). Control diluent cultured NCI-H520 cells secreted 240±80 pg/ml $PGE_2/1x10^5$ cells during a 24-h culture. Exposure of these cells to PC-SPES (2 or 4 μl/ml for 24 h) decreased the mean level of PGE_2 to 190±60 and 118±37 pg/ml/$1x10^5$ cells, respectively, suggesting that PC-SPES blocked COX-2 enzymatic activity.

In further experiments, NCI-H520 cells were transiently transfected with the wild-type COX-2 reporter construct, and cultured with either PC-SPES or control diluent. As expected, PC-SPES (2 μl/ml, 24 h) inhibited transcriptional regulation of COX-2 expression by approximately 60% as compared with control diluent treated cells (Fig. 2C). In addition, the effect of PC-SPES on level of COX-2 transcripts was studied using RAW264.7 murine macrophage-like cells; LPS (100 ng/ml, 6 h) increased COX-2 reporter activity by 5.0±2.0-fold and PC-SPES (2 μl/ml, 6 h) blunted this activity by about 50% (Fig. 2D). Recently, we purified oridonin from *Rabdosia rubescens*, one component of PC-SPES, by high performance liquid chromatography (HPLC) (13). The effect of oridonin on COX-2 reporter activity was investigated. As shown in Fig. 2C, oridonin (5 μg/ml, 24 h) inhibited approximately half the COX-2 reporter gene activity (Fig. 2C), suggesting that oridonin might represent one of the active component(s) of PC-SPES. Recently, Lot. 543129 of PC-SPES which was used in this study, was shown to contain warfarin (483 μg/g) and indomethacin (0.89 mg/g) (16). The final concentration of PC-SPES (2 μl/ml) utilized in our study, would theoretically contain approximately $2x10^{-6}$ M of these reagents. Importantly, even higher doses of warfarin ($1x10^{-5}$ M) and indomethacin ($1x10^{-5}$ M) did not inhibit transcriptional regulation of COX-2 expression in NCI-H520 cells (Fig. 2C).

Effect of PC-SPES on NF-κB activity in NSCLC cells. NCI-H520 cells were transiently transfected with a NF-κB reporter construct, and cultured with either PC-SPES or control diluent. PC-SPES (2 μl/ml, 24 h) inhibited NF-κB transcriptional activity by approximately 65±8% as compared with control diluent treated cells (Fig. 3A). Oridonin inhibited NF-κB trans-

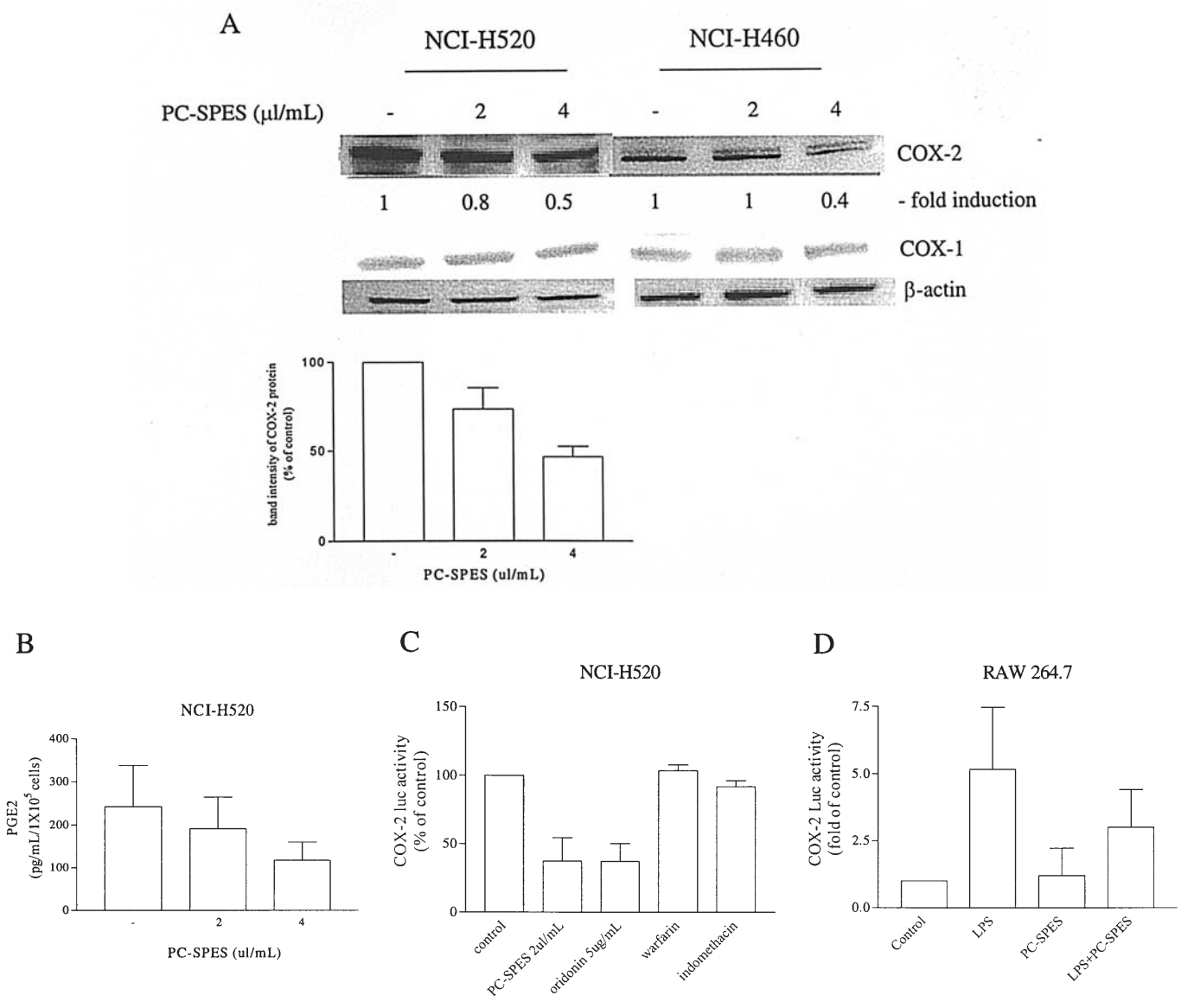

Figure 2. (A) Effect of PC-SPES on COX-2 protein in NCI-H520 and NCI-H460 cells. NCI-H520 and NCI-H460 cells were cultured in the presence of PC-SPES (2 or 4 μl/ml). After 24 h, cells were harvested, and subjected to Western blot analysis. Membranes were probed sequentially with anti-COX-2 and -1, as well as ß-actin antibody, and band intensities were measured using densitometry. A bar graph shows band intensities of COX-2 protein of NCI-H520 cells. Results represent the mean ± SD of three experiments done independently. (B) PC-SPES inhibits production of prostaglandin E_2 (PGE_2) in NCI-H520 cells. NCI-H520 cells ($2x10^5$/ml) were plated in 6-well plates (2 ml/well) and cultured either with or without PC-SPES (2 or 4 μl/ml). At 24 h, cell culture supernatants were collected and analyzed for levels of PGE_2 by ELISA. Results represent the mean ± SD of three experiments done in duplicates. (C) Effect of PC-SPES on wild-type COX-2 reporter activity in NCI-H520 cells. NCI-H520 cells were transfected with wild-type COX-2 promoter-luciferase reporter (0.5 μg/ml). Transfected cells were cultured either with or without PC-SPES (2 μl/ml), oridonin (5 μg/ml), warfarin (10-5 mol/l), or indomethacin (10^{-5} mol/l) for 24 h at which time luciferase activity was measured. pRL-SV40-luciferase (Renilla luciferase) vector was cotransfected for normalization. Results represent the mean ± SD of three experiments done in triplicates. (D) Effect of PC-SPES on LPS induced COX-2 reporter activity in RAW264.7 cells. RAW264.7 cells were transfected with COX-2 luciferase plasmid (0.5 μg). These cells were cultured with either PC-SPES (2 μl/ml) or control diluent (ethanol, 0.14%) for 1 h. At the end of the treatment, cells were washed twice with PBS and treated either with or without LPS (100 ng/ml) for 6 h at which time luciferase activity was measured. Results represent the mean ± SD of three experiments with triplicate dishes per experimental point. pRL-SV40-luciferase (Renilla luciferase) vector was cotransfected for normalization. LPS, lipopolysaccharide.

criptional activity by 37±8% (Fig. 3A). On the other hand, neither warfarin (10-5 mol/l) nor indomethacin (10^{-5} mol/l) affected NF-κB activity in NCI-H520 cells (Fig. 3A). Also, PC-SPES inhibited TNFα-induced NF-κB transcriptional activity in NCI-H520 cells; TNFα increased NF-κB transcriptional activity by about 4.8-fold and PC-SPES (2 μl/ml, 6 h) blunted this activity by 40% (Fig. 3B).

The effect of PC-SPES on NF-κB activity was further confirmed using an ELISA-based assay. Treatment of NCI-H520 cells with PC-SPES (2 μl/ml, 24 h) inhibited the NF-κB binding activity by 60% as compared with untreated control cells (Fig. 3C). As control, 100-fold molar excess of the wild-type NF-κB consensus oligonucleotides was added to the assay with lysate from untreated NCI-H520 cells. Binding was inhibited by at least 80%; in contrast, mutated NF-κB consensus oligonucleotides at the same molar excess were unable to inhibit binding (Fig. 3C), ascertaining the specificity of binding of NF-κB to its consensus binding site. Interestingly,

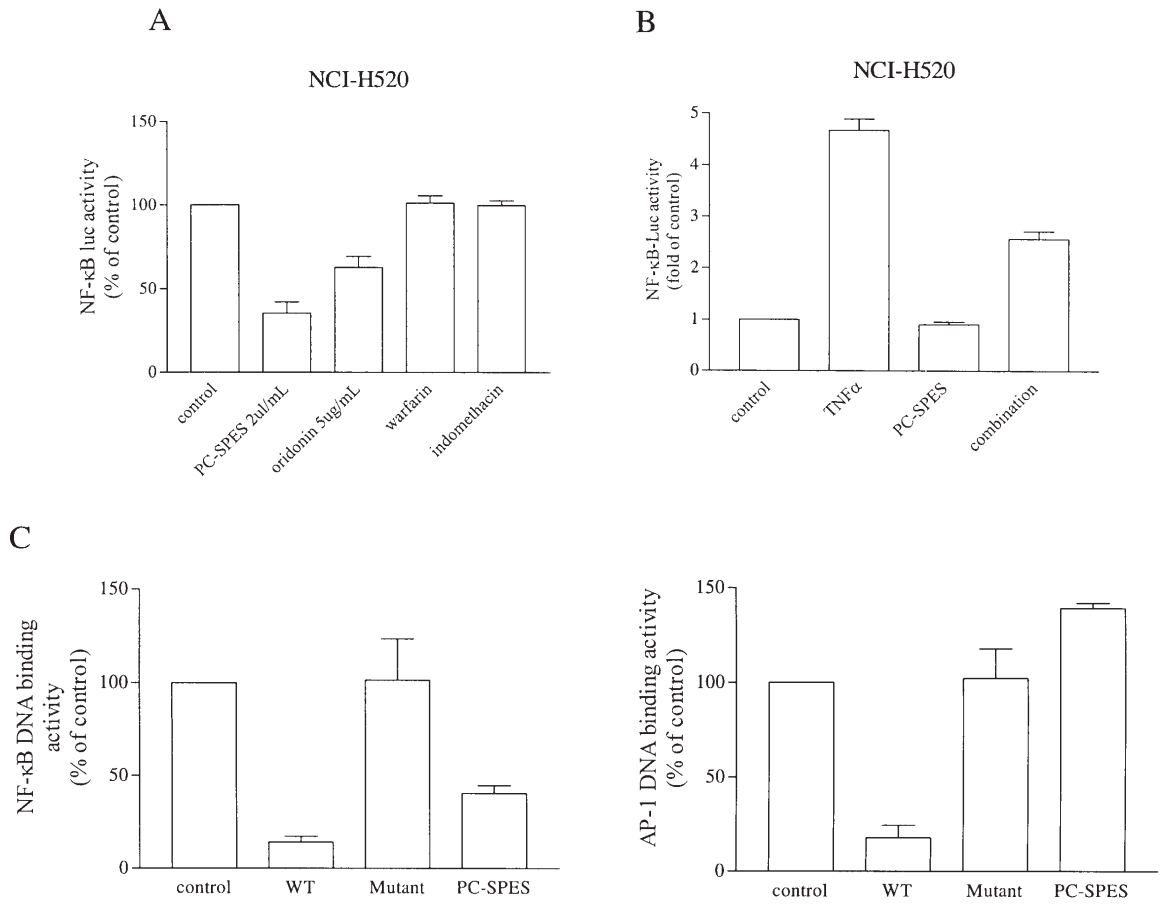

Figure 3. (A) Effect of PC-SPES on NF-κB reporter activity in NCI-H520 cells. NCI-H520 cells were transfected with NF-κB luciferase plasmid (0.5 μg/ml). Transfected cells were cultured either with or without PC-SPES (2 μl/ml), oridonin (5 μg/ml), warfarin (10^{-5} mol/l) or indomethacin (10^{-5} mol/l) for 24 h at which time luciferase activity was measured. pRL-SV40-luciferase (Renilla luciferase) vector was cotransfected for normalization. Results represent the mean ± SD of three experiments done in triplicates. (B) Effects of PC-SPES on TNFα induced NF-κB transcriptional activity in NCI-H520 cells. NCI-H520 cells were transfected with NF-κB nucleotide binding sequence luciferase reporter (0.5 μg). These cells were then cultured with either PC-SPES (2 μl/ml) or control diluent (ethanol, 0.14%) for 1 h. At the end of the treatment, cells were washed twice with PBS and treated either with or without TNFα (50 ng/ml) for 6 h at which time luciferase activity was measured. (C) Effect of PC-SPES on NF-κB and (D) AP-1 binding activity in NCI-H520 cells. NCI-H520 cells were plated in 6-well plates and cultured either with or without PC-SPES (2 μl/ml) for 24 h, nuclear protein was extracted and subjected to ELISA for measurement of NF-κB (C) and AP-1 (D) DNA binding activity. Results represent the mean ± SD of two experiments done in duplicates. WT, wild-type oligonucleotides; MT, mutated oligonucleotide.

PC-SPES (2 μl/ml, 24 h) slightly increased AP-1 binding activity (about 1.3-fold) as compared with control diluent treated cells under similar culture conditions (Fig. 3D) (p=0.04). These latter results are consistent with our previous observations showing that PC-SPES activated the JNK/c-Jun/AP-1 signal pathway in human prostate cancer LNCaP cells (17).

Effect of PC-SPES on levels of C/EBPs in NSCLC cells. C/EBPß binds to NF-IL6 sites and regulates the expression of numerous genes including COX-2. We therefore explored the effect of PC-SPES on C/EBPs in NSCLC cells. NCI-H520 and NCI-H460 cells were cultured in the presence of either 2 or 4 μl/ml of PC-SPES for 24 h. Protein was extracted and subjected to Western blot analysis. As shown in Fig. 4, both NCI-H520 and NCI-H460 cells constitutively expressed C/EBPß protein, and PC-SPES down-regulated levels of this transcription factor. For example, PC-SPES (4 μl/ml) reduced the amount of C/EBPß by 80% and 90% in NCI-H520 and NCI-H460 cells, respectively (Fig. 4). Also, we investigated the level of C/EBPα in the NSCLC cells. As reported previously, the expression of the p42 isoform of C/EBPα was barely detectable in lung cancer cells (18). PC-SPES (4 μl/ml) increased the p42 isoform by 3- or 4-fold in NCI-H520 and NCI-H460 cells, respectively (Fig. 4). On the other hand, the level of the dominant negative p30 isoform of C/EBPα was not significantly modulated.

Previous studies showed that non-steroidal anti-inflammatory drugs (NSAIDs) and acetylsalicylic acid (aspirin) blunted lipopolysaccharide (LPS)-induced expression of COX-2 in murine macrophages via inhibition of the C/EBPß transcriptional activity (19). Therefore, we explored whether indomethacin down-regulated levels of C/EBPß in NSCLC cells. Of note, even toxic amounts of indomethacin (10^{-4} mol/l) failed to down-regulate C/EBPß in NCI-H520 cells (Fig. 4B).

Effect of PC-SPES on the proliferation and apoptosis of NSCLC cells. To explore the anti-proliferative and proapoptotic effects of PC-SPES in NSCLC cells, NCI-H520, NCI-H460 and NCI-H1299 cells were cultured in the presence of various concentrations of PC-SPES. PC-SPES effectively inhibited the growth of these cells with an ED_{50s} of approximately

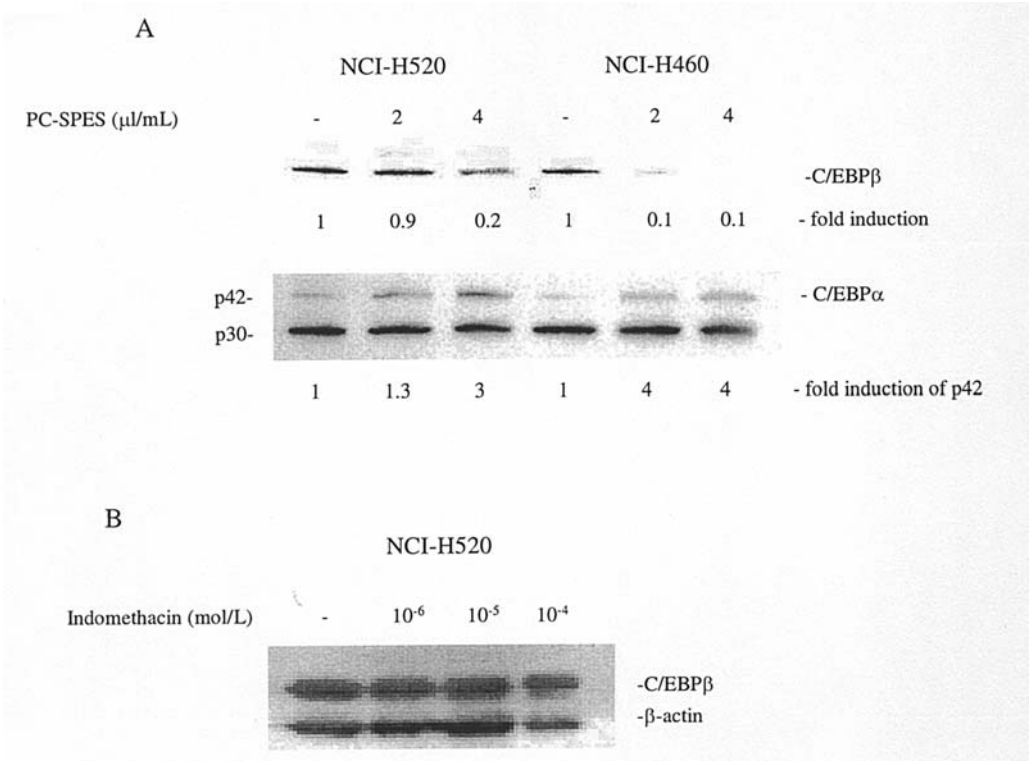

Figure 4. (A) Effect of PC-SPES on C/EBPs in NCI-H520 and NCI-H460 cells. NCI-H520 and NCI-H460 cells were cultured in the presence of PC-SPES (2 or 4 μl/ml). At 24 h, cells were harvested, subjected to Western blot analysis. Membranes were probed sequentially with anti-C/EBP-ß and -α antibodies, and band intensities were measured using densitometry. These studies were performed at least twice independently and similar results were obtained. (B) Effect of indomethacin on C/EBPß in NCI-H520 cells. NCI-H520 cells were cultured in the presence of indomethacin (10^{-6}-10^{-4} mol/l). At 24 h, cells were harvested and cellular lysates were subjected to Western blot analysis. Membranes were probed sequentially with anti-C/EBPß and ß-actin antibodies.

1.8 μl/ml for each of the cell lines as measured by MTT assay on the fourth day of culture (Fig. 5A). The final concentration of PC-SPES (Lot. 5431219) (4 μl/ml) used in our study would theoretically contain approximately $4x10^{-6}$ M of both warfarin and indomethacin. Importantly, even higher doses of both warfarin ($1x10^{-5}$ M) and indomethacin ($1x10^{-5}$ M) did not inhibit the growth of NCI-H520 cells (Fig. 5B). As shown in Fig. 5C, PC-SPES caused apoptosis of NCI-H520 cells in a dose- and time-dependent manner as measured by TUNEL assay. On the first day of culture, 1 and 4 μl/ml of PC-SPES induced a mean 3±1% and 10±2% of NCI-H520 cells to become apoptotic, respectively. The apoptotic population increased to a mean of 6±0.5% and 15±3%, respectively, on the second day of culture. Less than 1% of control cells were apoptotic on each day of culture (Fig. 5C).

The modulation of expression of the cell cycle 'checkpoint' proteins, $p21^{waf1}$ and p53 were examined by Western blot analysis. NCI-H520 and NCI-H460 cells constitutively expressed wild-type p53 protein. Exposure of these cells to PC-SPES (2 or 4 μl/ml, 24 h) resulted in p53 and $p21^{waf1}$ increasing 5- to 6-fold compared to untreated, control cells (Fig. 5D).

Discussion

This study demonstrated that NSCLC cells constitutively expressed COX-2 transcripts and protein. Both C/EBPß and NF-κB contributed to aberrant expression of COX-2 in NSCLC cells, and PC-SPES down-regulated levels of C/EBPß. Accumulating evidence suggests that C/EBPß can contribute to carcinogenesis and progression of several types of cancer. Forced expression of C/EBPß could transform normal mammary epithelial cells (20); and aberrant expression of C/EBPß was found in renal cell carcinoma, but not in the adjacent normal tissues (21). Also, murine C/EBPß deletional mice were resistant to development of activated ras mediated skin cancers (22). The role of C/EBPß in human lung cancer is yet to be explored.

Previously, other investigators showed that CRE and NF-IL6 sites played a critical role in aberrant expression of COX-2 in colon cancer cells (23). However, the importance of the NF-κB binding sites of this gene was unexplored. We found that mutation in the NF-κB site of the COX-2 promoter decreased reporter activity by 60% and mutation of both C/EBP (NF-IL6) binding sites decreased reporter activity by 90%. In addition, our study showed that the COX-2 reporter gene activity decreased only 23±14% when the CRE site of the COX-2 promoter was mutated (Fig. 3). Recently, another study investigating the molecular mechanism of COX-2 expression in murine lung tumor cell lines suggested the importance of the C/EBP but not the CRE site (14), which is consistent with our results. However, their study did not show the importance of NF-κB in the aberrant expression of COX-2. In their study, they employed deletional constructs of the promoter. On the other hand, we used promoter constructs that contained specific mutations of their NF-κB, C/EBP (NF-IL6), CRE and E-box sites. This may explain the discrepancy between our results and theirs. Another possible

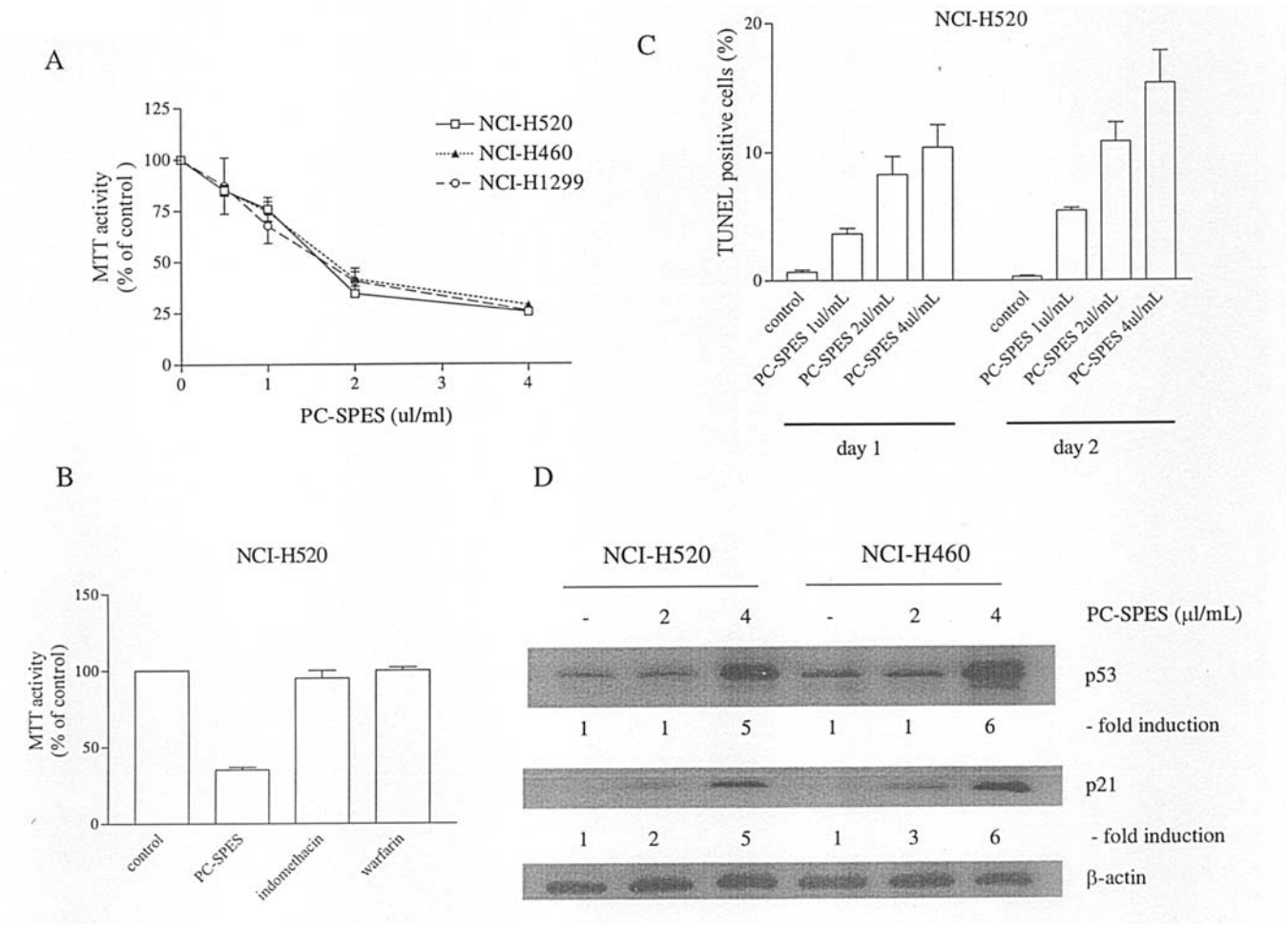

Figure 5. PC-SPES inhibits proliferation and induces apoptosis of non-small cell lung (NSCL) cancer cells. (A) NCI-H460, -H520, and -H1229 cells were placed in 96-well plates and cultured either with or without PC-SPES (0.5-4.0 μl/ml). After 4 days, the cells were treated with MTT for 4 h and its activity was measured. Results represent the mean ± SD of 3 experiments performed in triplicate. (B) NCI-H520 cells were plated in 96-well plates and cultured either with PC-SPES (2.0 μl/ml), indomethacin ($1x10^{-5}$ mol/l) or warfarin ($1x10^{-5}$ mol/l). After 4 days, the cells were treated with MTT for 4 h, and its activity was measured. Results represent the mean ± SD of 3 experiments done in triplicate. (C) NCI-H520 cells were plated in 8-chamber slides, cultured for 1 and 2 days with various concentrations of PC-SPES (1.0-4.0 μl/ml), and apoptosis was measured by TUNEL assay. Results represent the mean ± SD of 2 experiments done in triplicate. (D) NCI-H520 and -H460 cells were cultured in the presence of PC-SPES (2 or 4 μl/ml). At 48 h, cells were harvested and subjected to Western blot analysis. Membranes were probed sequentially with anti-$p21^{waf1}$ and -p53 antibodies, and band intensities were measured using densitometry.

explanation for this discrepancy could relate to the type of cells studied. They explored regulation of COX-2 in murine lung cancer cells and we used human lung cancer cells.

In our previous study, PC-SPES inhibited growth of COX-2 expressing and non-expressing colon cancer cells (15). Therefore, down-regulation of COX-2 can not totally explain the mechanism by which PC-SPES inhibits the growth of cancer cells. Recently, we explored the effect of PC-SPES on growth inhibition and induction of apoptosis of human prostate cancer LNCaP cells. We found that PC-SPES activated the JNK/c-Jun/AP-1 signal pathway (17). When this signal pathway was blocked by forced-expression of JNK interacting protein-1 (JIP), LNCaP cells became resistant to the growth inhibiting effects of PC-SPES. In the present study, PC-SPES increased DNA binding activity of AP-1 in NCI-H520 cells, suggesting that probably the JNK/c-Jun/AP-1 signal pathway also contributes to the growth inhibition and apoptosis of the cells cultured with PC-SPES.

Another possible mechanism by which PC-SPES inhibited the growth of many types of cancer cells could relate to its ability to down-regulate the level of tubulins in cancer cells; we and others showed that PC-SPES lowered the level of tubulins in colon cancer (10) as well as LNCaP cells (24-26) as these cells underwent apoptosis. Also, Bonham and colleagues found that PC-SPES could inhibit the polymerization of microtubules in LNCaP cells (26). Further studies are required to identify the active component(s) of PC-SPES that affect tubulins.

C/EBPα was shown to contribute to terminal differentiation of myeloid cells, hepatocytes, as well as adipocytes (27). Targeted inactivation of C/EBPα in mice demonstrated hyperproliferation of type II pneumocytes and abnormal alveolar structure, and histopathology of the liver displayed a structure resembling regenerative changes or hepatocellular carcinoma (28). We and others have identified mutations in the *C/EBPα* gene in individuals with acute myeloid leukemia, myelodysplastic syndrome and NSCLC: and these mutations disrupted normal function of C/EBPα (29,30). Recently, expression of C/EBPα was shown to be down-regulated in NSCLC cells compared to normal lung tissues, and forced

expression of the p42 isoform of C/EBPα reduced proliferation of NSCLC (18). Taken together, these findings reinforce the importance of C/EBPα in the regulation of cell growth and suggest that it may act as a tumor suppressor. We found that PC-SPES slowed cell growth of NSCLC and up-regulated level of C/EBPα in these cells. Further studies will explore the molecular mechanism by which the active component(s) of PC-SPES up-regulates C/EBPα.

We have purified baicalin and oridonin from PC-SPES, and have found that both can inhibit the growth of a wide variety of cancer cells including those from prostate, breast, and lung (13,31). Oridonin, but not baicalin, blunted the LPS-stimulated activity of NF-κB and expression of COX-2 in murine macrophage cells (unpublished data). Oridonin appears to be one of the active component(s) of PC-SPES.

In summary, this study provides the evidence that PC-SPES inhibited the proliferation and induced apoptosis of NSCLC cells. Also, PC-SPES down-regulated levels of COX-2 transcripts and protein in conjunction with inhibition of NF-κB transcriptional activity. It also decreased levels of C/EBPß and increased level of the p42 isoform of C/EBPα in NSCLC cells which may contribute to the ability of PC-SPES to inhibit the proliferation of these cells. PC-SPES might be useful in the adjuvant setting for the treatment of individuals with resected, non-small cell lung cancer as well as other types of cancer which have high COX-2 activity. Also, it might be useful for treatment of inflammatory diseases such as rheumatoid arthritis. Recently, warfarin, indomethacin, as well as DES have been found in some lots of PC-SPES (16). Lot. 5431219 used in this study, contained warfarin (483 μg/g) and indomethacin (0.89 mg/g), but not DES (16); each of these contaminates was at a concentration of about 2 or $4x10^{-6}$ M, respectively in our experiments. Therefore, we performed control studies and found that both of these compounds even at a very high concentration (10^{-5} mol/l), failed to affect either COX-2 (Fig. 2A) or NF-κB (Fig. 3A) activity, and did not down-regulate levels of C/EBPß (Fig. 4B) in NSCLC cells. Also, they did not inhibit the proliferation of NSCLC cells (Fig. 1). In addition, a new batch of PC-SPES did not contain either warfarin or indomethacin as determined by gas chromatography and mass spectrometry, but it had the same antiproliferative activity against NSCLC cells and did down-regulate COX-2 levels as seen by Western blot analysis (data not shown), similar to that reported here. Further studies are ongoing to isolate all of the active components of each herb that constitutes PC-SPES and look for additive and/or synergistic combinations.

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References

1. Smith WL and Marnett LJ: Prostaglandin endoperoxide synthase: structure and catalysis. Biochim Biophys Acta 1083: 1-17, 1991.
2. De Witt DL: Prostaglandin endoperoxide synthase: regulation of enzyme expression. Biochim Biophys Acta 1083: 121-134, 1991.
3. Suh N, Honda T, Finlay HJ, *et al*: Novel terpenoids suppress inducible nitric oxide synthase (iNOS) and inducible cyclo-oxygenase (COX-2) in mouse macrophages. Cancer Res 58: 717-723, 1998.
4. Rao CV, Indranie C, Simi B, Manning PT, Connor JR and Reddy BS: Chemopreventive properties of a selective inducible nitric oxide synthase inhibitor in colon carcinogenesis, administered alone or in combination with celecoxib, a selective cyclo-oxygenase-2 inhibitor. Cancer Res 62: 165-170, 2002.
5. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S and Dubois RN: Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 107: 1183-1188, 1994.
6. Gupta RA and Dubois RN: Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. Nat Rev Cancer 1: 11-21, 2001.
7. Di Paola RS, Zhang H, Lambert GH, *et al*: Clinical and biologic activity of an estrogenic herbal combination (PC-SPES) in prostate cancer. N Engl J Med 339: 785-791, 1998.
8. Small EJ, Frohlich MW, Bok R, *et al*: Prospective trial of the herbal supplement PC-SPES in patients with progressive prostate cancer. J Clin Oncol 18: 3595-3603, 2000.
9. Kubota T, Hisatake J, Hisatake Y, *et al*: PC-SPES: a unique inhibitor of proliferation of prostate cancer cells *in vitro* and *in vivo*. Prostate 42: 163-171, 2000.
10. Huerta S, Arteaga JR, Irwin RW, Ikezoe T, Heber D and Koeffler HP: PC-SPES inhibits colon cancer growth *in vitro* and *in vivo*. Cancer Res 62: 5204-5209, 2002.
11. Oshima M, Dinchuk JE, Kargman SL, *et al*: Inhibition of intestinal polyposis in $APC^{\Delta 716}$ knockout mice by inhibition of cyclooxygenase 2. Cell 87: 803-809, 1996.
12. Oshima M, Murai N, Kargman S, *et al*: Chemoprevention of intestinal polyposis in the $APC^{\Delta 716}$ mouse by refecoxib, a specific cyclooxygenase-2 inhibitor. Cancer Res 61: 1733-1740, 2001.
13. Ikezoe T, Chen S, Xian-Jun T, Heber D, Taguchi H and Koeffler HP: Oridonin induces growth inhibition and apoptosis of a variety of human cancer cells. Int J Oncol 23: 1187-1193, 2003.
14. Wardlaw SA, Zhang N and Belinsky SA: Transcriptional regulation of basal cyclooxygenase-2 expression in murine lung tumor-derived cell lines by CCAAT/enhancer-binding protein and activating transcription factor/cAMP response element-binding protein. Mol Pharmacol 62: 326-333, 2002.
15. Wadleigh DJ, Reddy ST, Kopp E, Ghosh S and Herschman HR: Transcriptional activation of the cyclooxygenase-2 gene in endotoxin-treated RAW 264.7 macrophages. J Biol Chem 275: 6259-6266, 2000.
16. Sovak M, Seligson AL, Konas M, Hajduch M, Dolezal M, Machala M and Nagourney R: Herbal composition PC-SPES for management of prostate cancer: identification of active principles. J Natl Cancer Inst 94: 1275-1281, 2002.
17. Ikezoe T, Chen S, Yang Y, Heber D, Taguchi H and Koeffler HP: PC-SPES: Molecular mechanism to induce apoptosis and down-regulate expression of PSA in LNCaP human prostate cancer cells. Int J Oncol 23: 1461-1470, 2003.
18. Halmos B, Huettner CS, Kocher O, Ferenczi K, Karp DD and Tenen DG: Down-regulation and antiproliferative role of C/EBPalpha in lung cancer. Cancer Res 62: 528-534, 2002.
19. Cieslik K, Zhu Y and Wu KK: Salicylate suppresses macrophage nitric-oxide synthase-2 and cyclo-oxygenase-2 expression by inhibiting CCAAT/enhancer-binding protein-beta binding via a common signaling pathway. J Biol Chem 277: 49304-49310, 2002.
20. Bundy LM and Sealy L: CCAAT/enhancer binding protein beta (C/EBPbeta)-2 transforms normal mammary epithelial cells and induces epithelial to mesenchymal transition in culture. Oncogene 13: 869-883, 2003.
21. Oya M, Horiguchi A, Mizuno R, Marumo K and Murai M: Increased activation of CCAAT/enhancer binding protein-beta correlates with the invasiveness of renal cell carcinoma. Clin Cancer Res 9: 1021-1027, 2003.
22. Zhu S, Yoon K, Sterneck E, Johnson PF and Smart RC: CCAAT/enhancer binding protein-beta is a mediator of keratinocyte survival and skin tumorigenesis involving oncogenic Ras signaling. Proc Natl Acad Sci USA 99: 207-212, 2002.
23. Shao J, Sheng H, Inoue H, Morrow JD and Dubois RN: Regulation of constitutive cyclooxygenase-2 expression in colon carcinoma cells. J Biol Chem 275: 33951-33956, 2000.

24. Bonham M, Arnold H, Montgomery B and Nelson PS: Molecular effects of the herbal compound PC-SPES: identification of activity pathways in prostate carcinoma. Cancer Res 62: 3920-3924, 2002.
25. Bigler D, Gulding KM, Dann R, Sheabar FZ, Conaway MR and Theodorescu D: Gene profiling and promoter reporter assays: novel tools for comparing the biological effects of botanical extracts on human prostate cancer cells and understanding their mechanisms of action. Oncogene 22: 1261-1272, 2003.
26. Bonham MJ, Galkin A, Montgomery B, Stahl WL, Agus D and Nelson PS: Effects of the herbal extract PC-SPES on microtubule dynamics and paclitaxel-mediated prostate tumor growth inhibition. J Natl Cancer Inst 94: 1641-1647, 2002.
27. Lekstrom-Himes J and Xanthopoulos KG: Biological role of the CCAAT/enhancer-binding protein family of transcription factors. J Biol Chem 273: 28545-28548, 1998.
28. Flodby P, Barlow C, Kylefjord H, Ahrlund-Richter L and Xanthopoulos KG: Increased hepatic cell proliferation and lung abnormalities in mice deficient in CCAAT/enhancer binding protein alpha. J Biol Chem 271: 24753-24760, 1996.
29. Pabst T, Mueller BU, Zhang P, *et al*: Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. Nat Genet 27: 263-270, 2001.
30. Gombart AF, Hofmann WK, Kawano S, *et al*: Mutations in the gene encoding the transcription factor CCAAT/enhancer binding protein alpha in myelodysplastic syndromes and acute myeloid leukemias. Blood 99: 1332-1240, 2002.
31. Ikezoe T, Chen SS, Heber D, Taguchi H and Koeffler HP: Baicalin is a major component of PC-SPES which inhibits the proliferation of human cancer cells via apoptosis and cell cycle arrest. Prostate 49: 285-292, 2001.