

## Research Article

# White Tea Extract Induces Apoptosis in Non–Small Cell Lung Cancer Cells: the Role of Peroxisome Proliferator-Activated Receptor- $\gamma$ and 15-Lipoxygenases

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## Abstract

Emerging preclinical data suggests that tea possess anticarcinogenic and antimutagenic properties. We therefore hypothesize that white tea extract (WTE) is capable of favorably modulating apoptosis, a mechanism associated with lung tumorigenesis. We examined the effects of physiologically relevant doses of WTE on the induction of apoptosis in non–small cell lung cancer cell lines A549 (adenocarcinoma) and H520 (squamous cell carcinoma) cells. We further characterized the molecular mechanisms responsible for WTE-induced apoptosis, including the induction of peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) and the 15-lipoxygenase (15-LOX) signaling pathways. We found that WTE was effective in inducing apoptosis in both A549 and H520 cells, and inhibition of PPAR- $\gamma$  with GW9662 partially reversed WTE-induced apoptosis. We further show that WTE increased PPAR- $\gamma$  activation and mRNA expression, concomitantly increased 15(S)-hydroxy-eicosatetraenoic acid release, and upregulated 15-LOX-1 and 15-LOX-2 mRNA expression by A549 cells. Inhibition of 15-LOX with nordihydroguaiaretic acid (NGDA), as well as caffeic acid, abrogated WTE-induced PPAR- $\gamma$  activation and upregulation of PPAR- $\gamma$  mRNA expression in A549 cells. WTE also induced cyclin-dependent kinase inhibitor 1A mRNA expression and activated caspase-3. Inhibition of caspase-3 abrogated WTE-induced apoptosis. Our findings indicate that WTE is capable of inducing apoptosis in non–small cell lung cancer cell lines. The induction of apoptosis seems to be mediated, in part, through the upregulation of the PPAR- $\gamma$  and 15-LOX signaling pathways, with enhanced activation of caspase-3. Our findings support the future investigation of WTE as an antineoplastic and chemopreventive agent for lung cancer. *Cancer Prev Res*; 3(9): 1132–40. ©2010 AACR.

## Introduction

Results from epidemiologic studies as well as laboratory experiments suggest that tea consumption confers protection against the development of chronic diseases, including cancer (1–6). Tea contains high levels of flavonoids, such as catechins and other polyphenols. These phytochemicals are thought to play key roles in mechanisms that might provide health benefits (7–9). Through these mechanisms, tea has shown significant chemopreventive effects in animal models of lung, skin, bladder, esophageal, and gastrointestinal cancers (10–14). For example,

green tea has been shown to inhibit lung tumor development in A/J mice treated with 4-(methylnitrosamino)-1-(3-pyridyl)-L-butanone, a potent nicotine-derived lung carcinogen found in tobacco (15). Although a majority of the work evaluating the antineoplastic effects of tea has been done on green tea, the potential health benefits of white tea and its advantage over green tea have become increasingly recognized in recent years.

White tea is a rare tea that comes from the same plant (*Camellia sinensis*) as green and black teas. The descriptive term “white” stems from the high proportion of silvery buds harvested from the plants to produce the tea. White tea is the least processed of all teas, as it is picked, then rapidly steamed and dried, without fermentation, roasting, or subjected to a prior withering stage. Green tea is produced when freshly harvested leaves are subjected to withering, then pan-fried/steamed prior to rolling/shaping, and drying. Black tea, which represents the largest percentage of the total tea consumption worldwide, is produced by following some of the processing steps used for green tea, with critical differences in which the leaves are bruised, crushed, or broken, thus allowing the polyphenol oxidases in the leaf to convert the endogenous catechins into theaflavins, thearubigins, and other complex polyphenols.

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The degree of protection against mutagenesis and carcinogenesis seems to be related to the extent of processing, because the catechins are generally considered to be more active based on their antioxidant and other protective properties. As such, the minimal processing of white tea yields a higher concentration of polyphenol antioxidants.

To date, most of the studies evaluating the effects of tea on lung cancer have been focused on green tea, and a review of the literature yielded no published work involving WTE. On the basis that white tea has been shown to possess higher antimutagenic potency than green tea (16), we hypothesize that WTE will be effective in modulating mechanisms associated with lung tumorigenesis. We evaluated the effects of WTE on apoptosis in non-small cell lung cancer (NSCLC) cells, because apoptosis is an essential regulatory process that maintains homeostasis in normal epithelial cells, and resistance to apoptosis is a critical mechanism that confers the malignant phenotype. As such, restoring apoptosis might represent an important anticarcinogenic mechanism mediated by WTE.

Emerging data continues to link carcinogenesis to inflammatory events involving the eicosanoid metabolic pathways. Arachidonic acid, released from membrane phospholipids upon cell stimulation, is converted to prostanooids by cyclooxygenases, to leukotrienes by 5-lipoxygenase (5-LOX), and to 15(S)-hydroxy-eicosatetraenoic acid (15-HETE) by 15-LOXs (17). Overexpression of cyclooxygenase-2 and prostaglandin  $E_2$  are associated with a variety of well-established lung cancer risk factors, including resistance to apoptosis (18). Preclinical data suggests that in addition to the prostaglandins, the LOX signaling pathways might play a significant role in lung cancer tumorigenesis. Shankaranarayanan and colleagues showed that 15-HETE bound to peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) nuclear receptors and induced apoptosis in A549 cells. Moreover, pretreatment of cells with nordihydroguaiaretic acid (NGDA), a 15-LOX inhibitor, prevented PPAR- $\gamma$  activation and apoptosis (19). Shappell and colleagues also found that 15-LOX-2-derived 15-HETE might constitute an endogenous ligand for PPAR- $\gamma$  in the prostate and that loss of this pathway by reduced expression of 15-LOX-2 might contribute to increased proliferation and reduced differentiation in prostate carcinoma (20). We therefore hypothesized that WTE is capable of inducing apoptosis in human NSCLC via modulation of the 15-LOX pathway.

We show that WTE is effective in inducing apoptosis in both A549 and H520 cells. We also show that WTE significantly increases 15-HETE release, upregulates 15-LOX-1 and 15-LOX-2, as well as PPAR- $\gamma$  mRNA expression, and enhances activation of PPAR- $\gamma$  by A549 cells. These mechanisms in turn increase caspase-3 activation. Our findings support the future investigation of WTE as a chemopreventive agent for lung cancer.

## Materials and Methods

### Cell culture

As models to evaluate the effect of WTE on lung cancer, the human lung adenocarcinoma cell lines, A549 and H520 (American Type Culture Collection), were studied *in vitro*. Cells were maintained as monolayers in an atmosphere of 5%  $CO_2$  in air at 37°C in 25  $cm^2$  tissue culture flasks containing 5.0 mL of RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, 0.1 mg/mL of streptomycin, and 2 mmol/L of glutamine (JRH Biosciences) for A549 cells, and RPMI 1640 with 2 mmol/L L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mmol/L HEPES, and 1.0 mmol/L sodium pyruvate; 90% fetal bovine serum and 10% for H520 cells. A549 or H520 cells ( $0.1 \times 10^6$ ) were plated and incubated at 37°C for 2 hours. Different doses of WTE containing 3.5, 5, 7, and 10  $\mu$ g/mL of epigallocatechin-3-gallate (EGCG) were added and cells were incubated at 37°C overnight for 16 to 18 hours. The dose range of WTE was chosen based on physiologically relevant doses that have been reported in previous studies (21–23). To ascertain the involvement of PPAR- $\gamma$ , 15-LOX, and caspase-3 in our model systems, PPAR- $\gamma$  inhibitor (GW9662; Cayman Chemical), exogenous 15-HETE (Cayman Chemical), 15-LOX inhibitors [caffeic acid (Biomol Research Laboratory Inc.) and NGDA (Sigma)], or caspase-3 inhibitor Z-DEVD-FMK (R&D Systems), were added 1 hour prior to the addition of WTE. Experiments with dose titrations of each of these inhibitors were first carried out to determine the optimal inhibitory dose without compromising cell viability. Primary normal human bronchial epithelial (NHBE) cells (Cambrex) and human bronchoalveolar lavage cells, obtained from chemoprevention trials as previously described (24) were used as controls. NHBE cells were maintained according to the instructions of the manufacturer. NHBE cells ( $0.5 \times 10^6$ ) were plated in wells and incubated at 37°C overnight. Bronchoalveolar lavage cells ( $0.5 \times 10^6$ ) were plated in serum-free X-vivo medium (Bio Whittaker) for 2 hours. Similar doses of WTE were then added and cells were incubated at 37°C overnight for 16 to 18 hours.

All conditioned culture supernatants, total RNA, or total protein were harvested and stored at -80°C until analysis. Samples of mRNA were collected using the QIAGEN RNeasy Mini Kit (Qiagen Inc.). Total proteins were obtained by scrapping the cells off the plate in Laemmli buffer and homogenized by repeatedly passing the cells through a 25-gauge syringe.

### PPAR- $\gamma$ shRNA plasmid transfection

Stable transfection of PPAR- $\gamma$  shRNA plasmid into A549 cells was achieved using the PPAR- $\gamma$ -specific SureSilencing shRNA Plasmid Kit (SA Biosciences). Transfected clones were selected using various concentration of G418 according to the instructions of the manufacturer. Selected clones attained 80% inhibition of PPAR- $\gamma$  mRNA expression confirmed by quantitative PCR.

### Preparation of tea extract and quantification of tea catechin level

WTE and green tea extract (GTE) were purchased from commercially available sources. To ensure that the same amount of WTE from various vendors were used for comparison experiments, a sample of the materials was first analyzed at an independent, commercial laboratory (Industrial Laboratory) for confirmation. Stock solutions of WTE standardized to EGCG and total catechin contents were made by dissolving each extract with double-distilled water. Aliquots of the stock were stored at  $-80^{\circ}\text{C}$  and used only once for each set of experiments.

### Quantification of apoptosis in conditioned cells

For quantification of apoptosis, the cell death detection ELISA kits (Roche) were used according to the instructions of the manufacturer to quantify apoptosis in the conditioned cells, as measured by specific determination of mononucleosomes and oligonucleosomes in the cytoplasmic fraction of conditioned cell culture lysates.

### Real-time quantitative PCR

First-strand cDNA was synthesized using 2.5  $\mu\text{g}$  of total RNA (DNase treated) in a 50  $\mu\text{L}$  reverse transcriptase reaction mixture from the iScript cDNA Synthesis Kit (Bio-Rad). A region of the  $\beta$ -actin, 15-LOX-1, 15-LOX-2, and PPAR- $\gamma$  mRNA were amplified using specific primer pairs (IDT):  $\beta$ -actin, sense 5'-GTACCACTGGCATCGTGAT-3', antisense 5'-ATCTTCATGAGGTAGTCAGTCA-3'; 15-LOX-1, sense 5'-GGGCAAGGAGACAGAACTCAA-3', antisense 5'-GCACAGATCCAGTTGCGAGAA-3'; 15-LOX-2, sense 5'-TCGCCTCCAGTTCCTGAA-3', antisense 5'-AGTGACGGGGAAGTCTTTGG-3'; PPAR- $\gamma$ , sense 5'-CCTATTGACCCAGAAAGCGATT-3', antisense 5'-CATTACGGAGAGATCCACGGA-3'.

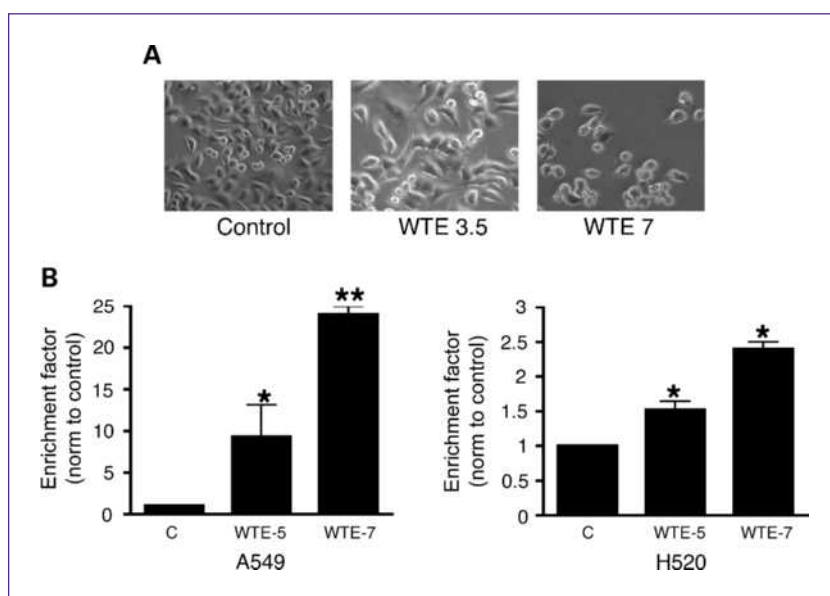
The length of the cDNA-amplified fragments were  $\beta$ -actin (136 bp), 15-LOX-1 (122 bp), 15-LOX-2 (80 bp), and PPAR- $\gamma$  (135 bp). All real-time PCR reactions were performed in a 25  $\mu\text{L}$  mixture containing 1/25 volume of cDNA preparation (2  $\mu\text{L}$ ) and  $1\times$  iQ SYBR Green Supermix (Bio-Rad). The PCR amplification protocol consists of 1 minute denaturing ( $95^{\circ}\text{C}$ ), 1 minute annealing ( $60^{\circ}\text{C}$ ), and 1 minute extension ( $72^{\circ}\text{C}$ ) for a total of 35 cycles. Real-time quantifications were performed using the BIO-RAD iCycler iQ system (Bio-Rad). The values were first normalized to  $\beta$ -actin, then to the control values for each experiment. Data are depicted in fold changes normalized to controls. The fluorescence threshold value was calculated using the iCycle iQ system software (PCR efficiency ranged from 90% to 97%). The quantitative PCR reactions for cyclin-dependent kinase inhibitor 1A (CDKN1A) gene were performed using reagents,  $\beta$ -actin and CDKN1A primers from SA Biosciences, according to the instructions of the manufacturer.

### Quantification of 15-HETE and 13-S-HODE

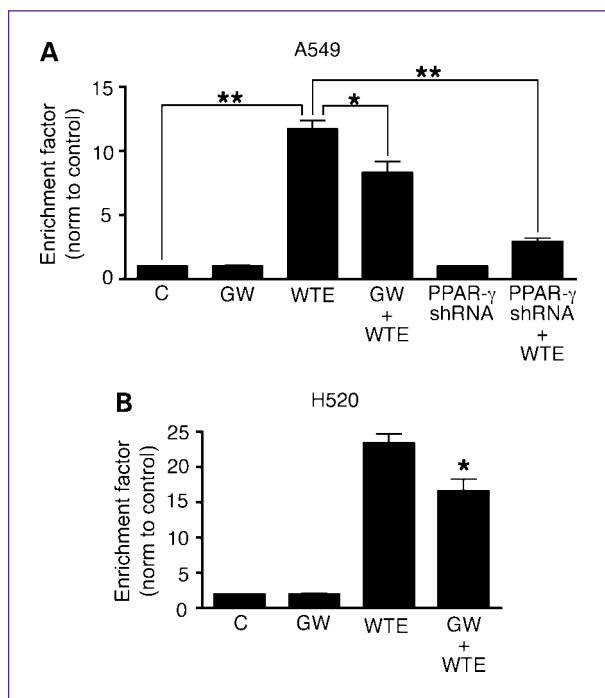
Measurement of 15-HETE concentrations in conditioned culture supernatants were performed to assess the effects of WTE on modulating the production of this arachidonic acid metabolite, using enzyme-specific immunoassay (EIA) according to the EIA kit protocol from Cayman Chemical. Measurements of 13(S)-hydroxyoctadecadienoic acid (13-S-HODE) levels in the same conditioned culture supernatants were performed using EIA according to the instructions of the manufacturer (Assay Design).

### Immunofluorescence of activated PPAR- $\gamma$

Immunofluorescence analyses were performed using A549 cells directly cultured in eight-well chamber slide (Nalge Nunc International). After 8 hours of adherence,



**Fig. 1.** A, WTE induced morphologic changes in A549 cells in a dose-dependent manner. A549 cells were incubated with varying doses of WTE. Representative photomicrographs of conditioned A549 cell culture following 17 h of incubation: left, control; middle, WTE containing 3.5  $\mu\text{g}/\text{mL}$  of EGCG; right, WTE containing 7  $\mu\text{g}/\text{mL}$  of EGCG. B, quantification of apoptosis in conditioned A549 and H520 cells by cell death detection ELISA. WTE induced apoptosis in A549 and H520 cells in a dose-responsive manner, as measured by specific determination of mononucleosomes and oligonucleosomes in the cytoplasmic fraction of cell culture lysates. WTE 5, WTE containing 5  $\mu\text{g}/\text{mL}$  of EGCG; WTE 7, WTE containing 7  $\mu\text{g}/\text{mL}$  of EGCG. The mean  $\pm$  SD absorbance values at 405 nm are reported. Columns, mean; bars, SE ( $n = 3$ ). \*,  $P < 0.05$ . \*\*,  $P < 0.01$ .



**Fig. 2.** Inhibition of PPAR- $\gamma$  with GW9662 significantly abrogated WTE-induced apoptosis in both A549 (A) and H520 cells (B). Inhibition of PPAR- $\gamma$  mRNA expression with PPAR- $\gamma$  shRNA plasmid also abrogated WTE-induced apoptosis in A549 cells (A). Columns, mean; bars, SE ( $n = 3$ ). \*,  $P < 0.05$ . \*\*,  $P < 0.01$ .

A549 cells were conditioned with WTE and/or NGDA for 13 hours. Cells were then fixed with 3.7% paraformaldehyde for 30 minutes and subjected to triple-labeling of PPAR- $\gamma$  DNA. A549 cells were incubated with 1:50 mouse monoclonal anti-PPAR- $\gamma$  antibody (Santa Cruz Biotechnology) for 2 hours, 1:500 Cy3-conjugated AffiniPure goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratory) for 30 minutes and 1:10,000 dilution of 4',6-diamidino-2-phenylindole stock solution for 5 minutes. Between each incubation step, the cover glass was rinsed three times with cold PBS. The slides were then mounted in PermaFluor Mountant medium (Thermo Electron Corp.) for fluorescence microscopic examination. Images were collected using an Olympus U-DO microscope equipped with an Olympus Imaging U-CNAD 3 digital camera.

#### Quantification of caspase-3 activity

For quantification of caspase-3 activity, the caspase-3/CPP32 Colorimetric Assay Kits (BioVision) were used. The assay is based on spectrophotometric detection of the chromophore *p*-nitroanilide after cleavage from the labeled substrate DEVD-*p*-nitroanilide. The *p*-nitroanilide light emission was quantified using a microtiter plate reader at 405 nm. Freshly harvested cell extracts from conditioned cultures were assayed according to the instructions of the manufacturer.

#### Statistical analysis

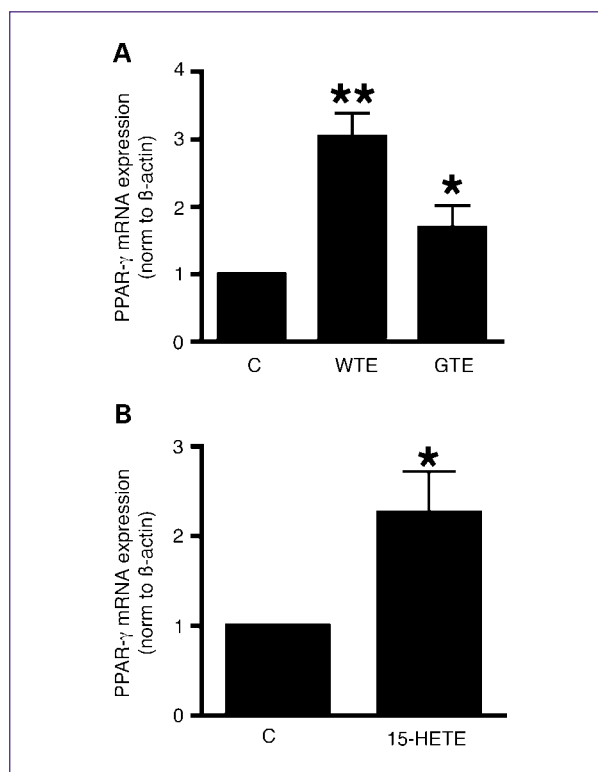
Data are expressed as the mean  $\pm$  SEM in all circumstances in which mean values were compared. Data were analyzed by paired Student's *t* test and/or ANOVA. Batch analyses were performed for each subject/comparison group to eliminate interassay variability. Differences are considered significant when  $P < 0.05$ .

## Results

### WTE induces apoptosis in NSCLC cells

To evaluate the potential of WTE on apoptosis induction, we examined the effects of WTE from various commercially available sources on inducing apoptotic cell death in A549 cells and H520 cells. Treatment with WTE increases apoptosis in both A549 and H520 cells in a dose-dependent manner (Fig. 1A and B).

To determine whether or not the observed WTE-induced changes were due to nonspecific, direct cytotoxicity, similar experiments were performed on human bronchoalveolar lavage cells and NHBE cells. Treatment of human bronchoalveolar lavage and NHBE cells with WTE at the same



**Fig. 3.** A and B, WTE (containing 7  $\mu$ g/mL of EGCG), GTE (containing 7  $\mu$ g/mL of EGCG), and 15-HETE (3  $\mu$ mol/L) all significantly upregulated PPAR- $\gamma$  mRNA expression in A549 cells following 17 h of incubation. When compared with the same dose of GTE with comparable catechin content, WTE was significantly more effective than GTE in the upregulation of PPAR- $\gamma$  transcripts. Columns, mean; bars, SE ( $n = 3$ ). \*,  $P < 0.05$ . \*\*,  $P < 0.01$ .

**Table 1. Catechin compositions in WTE and GTE**

	Total catechin (mg/mL)	EGCG (mg/mL)
WTE	1.73	0.93
GTE	1.67	0.98

doses does not result in significant morphologic changes nor in an increase in apoptosis (data not shown).

#### Inhibition of PPAR- $\gamma$ abrogated WTE-induced apoptosis in both A549 and H520 cells

To determine whether WTE-induced apoptosis is mediated via the PPAR- $\gamma$  pathway, we pretreated A549 and H520 cells with GW9662, a PPAR- $\gamma$  inhibitor, followed by conditioning with WTE (containing 5  $\mu$ g/mL of EGCG). Inhibition of PPAR- $\gamma$  with GW9662 significantly abrogated WTE-induced apoptosis in both A549 and H520 cells

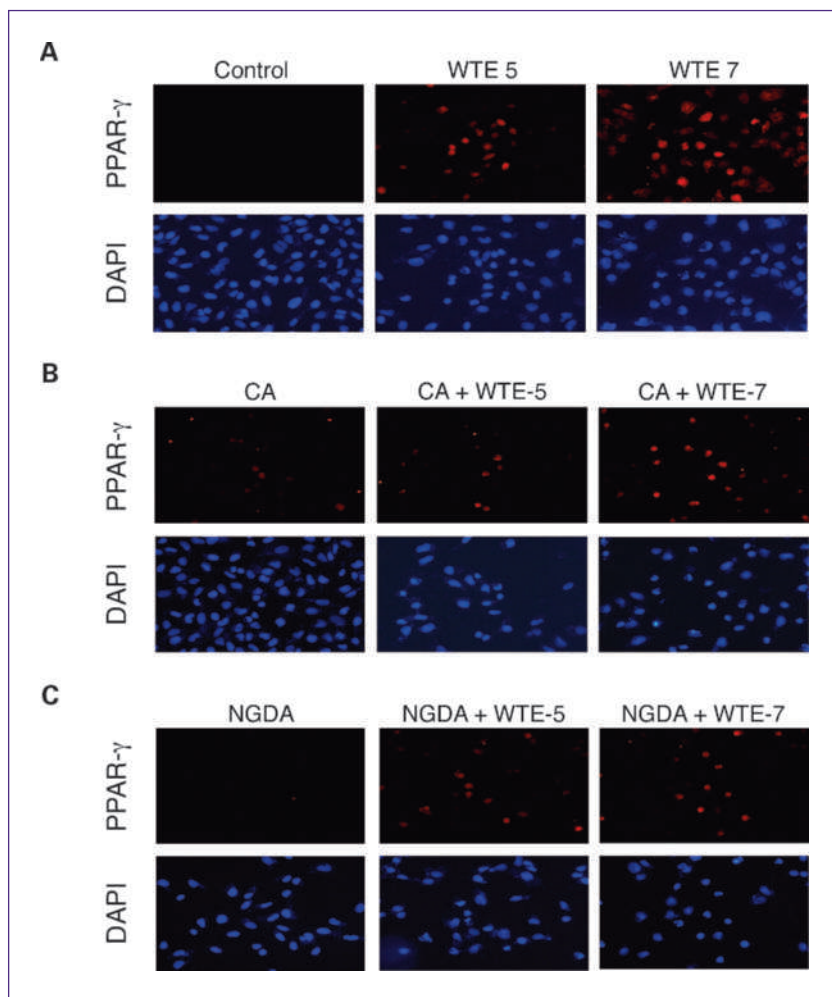
(Fig. 2A and B). Inhibition of PPAR- $\gamma$  mRNA expression with PPAR- $\gamma$  shRNA plasmid also abrogated WTE-induced apoptosis in A549 cells (Fig. 2A).

#### WTE, GTE, and exogenous 15-HETE all induce PPAR- $\gamma$ mRNA expression in A549 cells

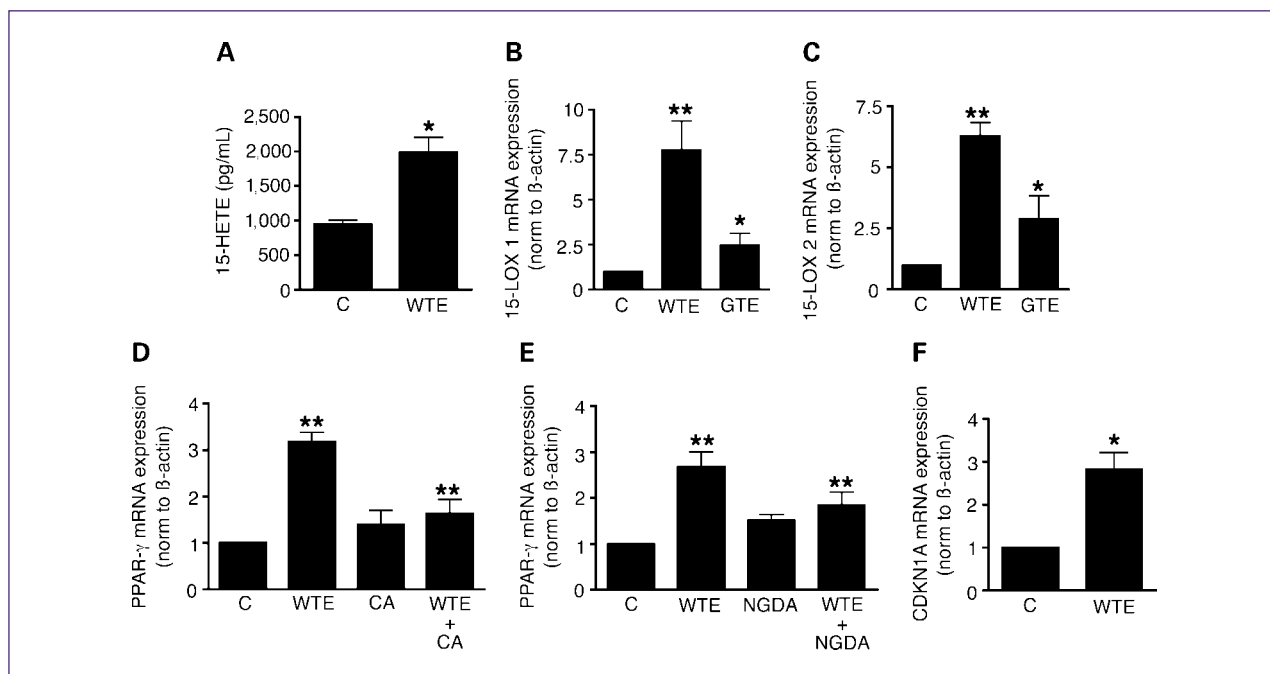
We then looked at the effects of WTE, GTE, and exogenous 15-HETE on PPAR- $\gamma$  mRNA expression in A549 cells. WTE (containing 7  $\mu$ g/mL of EGCG), GTE (containing 7  $\mu$ g/mL of EGCG), and 15-HETE (3  $\mu$ mol/L) all significantly upregulated PPAR- $\gamma$  mRNA expression in A549 cells after 17 hours of incubation (Fig. 3A and B). Interestingly, when compared with the same dose of GTE with similar compositions of catechin (Table 1), although WTE and GTE both induced PPAR- $\gamma$  mRNA expression in A549 cells, WTE was significantly more effective than GTE in the upregulation of these transcripts.

#### WTE induces PPAR- $\gamma$ activation in A549 cells

To evaluate the effects of WTE on PPAR- $\gamma$  activation, immunofluorescence analysis of PPAR- $\gamma$  in conditioned



**Fig. 4.** To evaluate the effects of WTE on PPAR- $\gamma$  activation, immunofluorescence analysis of PPAR- $\gamma$  in conditioned cells was performed. Nuclear translocation is a necessary step for, and is a marker of, PPAR- $\gamma$  activation (37, 38). Treatment with WTE (containing 5 or 7  $\mu$ g/mL of EGCG) significantly increased translocation of PPAR- $\gamma$  from the cytoplasm to the nucleus in a dose-dependent manner and pretreatment with caffeic acid (CA) or NGDA abrogated the WTE-induced nuclear staining of PPAR- $\gamma$ . Original magnification,  $\times 20$  ( $n = 2$ ).



**Fig. 5.** WTE (containing 7  $\mu\text{g/mL}$  of EGCG) significantly increases 15-HETE production (A) and 15-LOX-1 and 15-LOX-2 expression (B and C) by A549 cells. Again, when compared with the same dose of GTE with comparable composition of catechin content, WTE was significantly more effective than GTE in the upregulation of these transcripts. Preconditioning of A549 cells with caffeic acid (CA), (D), as well as NGDA (E), abrogates WTE (containing 5  $\mu\text{g/mL}$  of EGCG)-induced upregulation of PPAR- $\gamma$  mRNA expression. Columns, mean; bars, SE ( $n = 3$ ). WTE also significantly upregulated CDKN1A mRNA expression in A549 cells (F). Columns, mean; bars, SE ( $n = 3$ ). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

cells was performed. Treatment with WTE (containing 5 and 7  $\mu\text{g/mL}$  of EGCG) significantly increased nuclear staining of PPAR- $\gamma$  in a dose-dependent manner, and pretreatment with the 15-LOX inhibitors, caffeic acid (2.2  $\mu\text{mol/L}$ ) or NGDA (20  $\mu\text{mol/L}$ ), abrogated the WTE-induced nuclear staining of PPAR- $\gamma$ . Images were taken using a Nikon Eclipse E400 microscope at 20 $\times$  magnification (Fig. 4).

#### WTE significantly increases 15-HETE production and 15-LOX-1 and 15-LOX-2 expression by A549 cells

We show that WTE (containing 5  $\mu\text{g/mL}$  of EGCG) significantly increases 15-HETE production (Fig. 5A), 15-LOX-1 and 15-LOX-2 mRNA expression, corresponding to the increase in apoptotic cell death (Fig. 5B and C). Again, when compared with the same dose of GTE with comparable catechin content, WTE was significantly more effective than GTE in the upregulation of these transcripts.

#### Inhibition of 15-LOX abrogated the WTE-induced upregulation of PPAR- $\gamma$ mRNA expression in A549 cells

When A549 cells were preconditioned with the 15-LOX inhibitors, caffeic acid, as well as NGDA, WTE (containing 5  $\mu\text{g/mL}$  of EGCG) loses its ability to upregulate PPAR- $\gamma$  mRNA expression in A549 cells (Fig. 5D and E).

#### WTE significantly upregulated CDKN1A mRNA expression in A549 cells

WTE (containing 5  $\mu\text{g/mL}$  of EGCG) also significantly upregulated the mRNA expression of CDKN1A, a target gene of PPAR- $\gamma$  in A549 cells (Fig. 5F).

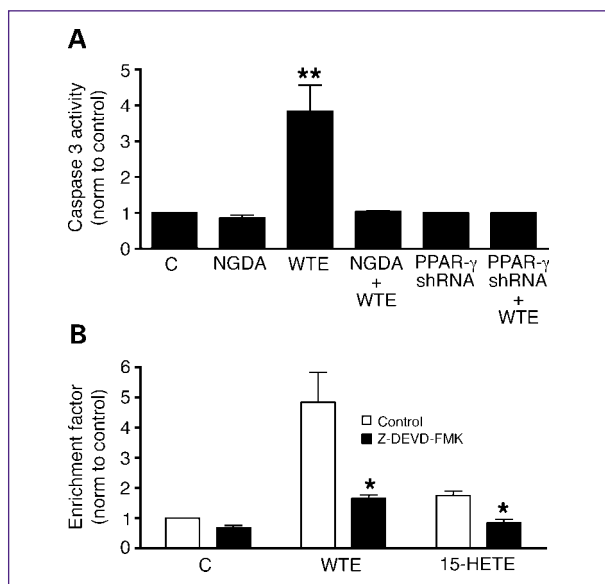
#### WTE induces caspase-3 activation in A549 cells

To determine whether the WTE-induced apoptosis in A549 cells involves caspase-3 activation, the activities of caspase-3 in conditioned cells were measured. Treatment with WTE significantly increased caspase-3 activity; inhibition of 15-LOX with NGDA and PPAR- $\gamma$  with PPAR- $\gamma$  shRNA both abrogated WTE-induced activation of caspase-3 (Fig. 6A). Inhibition of caspase-3 with DEVD-FMK (100  $\mu\text{mol/L}$ ) abrogated the WTE-induced apoptosis (Fig. 6B).

#### Discussion

In this study, we report the effect of WTE on inducing apoptosis in NSCLC cells. We further show, for the first time, the roles of novel signaling pathways—PPAR- $\gamma$  and 15 LOX in mediating tea-induced apoptosis, which involves the activation of caspase-3.

PPARs are nuclear hormone receptors that act as ligand-activated transcription factors. They are involved in lipid transport and metabolism (25). The transcriptional activity of PPARs is regulated by fatty acid binding. PPARs



**Fig. 6.** WTE induces caspase-3 activation in A549 cells. WTE (containing 5  $\mu$ g/mL of EGCG) induces apoptosis in A549 cells via caspase-3 activation. Inhibition of 15-LOX with NGDA and PPAR- $\gamma$  with PPAR- $\gamma$  shRNA (A) both abrogated WTE-induced activation of caspase-3. Inhibition of caspase-3 with DEVD-FMK abrogated the WTE-induced apoptosis (B). Columns, mean; bars, SE ( $n = 3$ ). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

can bind to DNA only as a heterodimer with the retinoid X receptor. Three PPAR isotypes have been identified:  $\alpha$ ,  $\beta$ , and  $\gamma$ . PPAR- $\gamma$  stimulates lipolysis of circulating triglycerides and the subsequent uptake of fatty acids into adipose cells. As a nuclear receptor which plays a pivotal role in regulating adipocyte differentiation, glucose and lipid homeostasis, and intracellular insulin-signaling events, PPAR- $\gamma$  has been receiving growing interest for its involvement in carcinogenesis. Reduced PPAR- $\gamma$  expression within the tumor is associated with poor prognosis in patients with lung cancer. Synthetic PPAR- $\gamma$  agonists such as the thiazolidinedione class of antidiabetic drugs could inhibit the growth of NSCLC cells *in vitro*, and block tumor progression in xenograft models (26). Preclinical studies in NSCLC, colon, and prostate cancer cell lines have also implicated the involvement of PPAR- $\gamma$  in mediating apoptosis, with 15-HETE acting as its ligand (19, 20, 27). Our data indicates that the WTE-induced apoptosis in NSCLC cells is, in part, mediated by increased 15-HETE production, 15-LOX-1, 15-LOX-2, and PPAR- $\gamma$  expression, resulting in increased PPAR- $\gamma$  activation.

LOXs are lipid-peroxidizing enzymes that are categorized according to their position in the oxygenation of arachidonic acid, e.g., 15-LOX oxygenates arachidonic acid at C-15 (28). 15-LOX-1 metabolizes not only linoleic acid, the predominant polyunsaturated fatty acid in human diet, to 13-S-HODE but also arachidonic acid to 15-HETE. Numerous studies on LOX expression in human tumors, as well as in animal models, indicate different roles of

the distinct LOX in carcinogenesis (29). Data suggest that the 5-LOX and platelet type 12-LOX exhibit procarcinogenic activities, whereas 15-LOX-1 and 15-LOX-2 might suppress carcinogenesis. 15-HETE is the most abundant eicosanoid in human bronchi (30). In addition to playing a role in apoptosis, 15-HETE has been shown to be chemotactic and chemokinetic for polymorphonuclear leukocytes and vascular smooth muscle cells. Moreover, 15-HETE also acts as a second messenger in angiotensin II-induced aldosterone production. Evidence suggests many other biological activities, including hyperphosphorylation of several major cytoskeletal proteins and enhancing surface expression of integrin receptors (31). Because 15-LOX-1 is known to preferentially metabolize linoleic acid to 13-S-HODE (29), we evaluated 13-S-HODE levels in the culture supernatants and found that they were below the detection limit of the EIA (data not shown).

To further substantiate the involvement of PPAR- $\gamma$ , we measured the effects of WTE on CDKN1A mRNA expression, a known target gene of PPAR- $\gamma$  (32), and found that WTE significantly upregulated CDKN1A mRNA expression. The *CDKN1A* gene encodes the P21 (WAF1) protein, which functions as a regulator of cell cycle progression. This protein has been reported to be specifically cleaved by caspase-3-like caspases, which might be instrumental in the execution of apoptosis following caspase activation (33).

Caspase-3 is an intracellular cysteine protease that exists as a proenzyme, becoming activated during the sequence of events associated with apoptosis. Detection of caspase-3 activity in cells is an important means for evaluating the mechanisms of apoptosis induced by a wide variety of apoptotic signals (34). In our study, we found that WTE-induced apoptosis in NSCLC cells involves the activation of caspase-3, and inhibition of both PPAR- $\gamma$  and 15-LOX abrogated the effects of WTE on caspase-3 activities, indicating that both PPAR- $\gamma$  and 15-LOX pathways are involved in caspase-3 activation.

EGCG, the major constituent of tea catechins, which is found in higher concentrations in white tea than in green tea, has been shown to inhibit benzo(a)pyrene-induced mutagenesis in the lung of *rps1* transgenic mice (35). Santana-Rios and colleagues reported that white tea exhibited greater antimutagenic activity in the *Salmonella* assay compared with green tea (16). In additional studies, white tea inhibits 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine-induced colonic aberrant crypt foci in male F344 rats by altering the expression of carcinogen-metabolizing enzyme (14). White tea also suppressed intestinal polyp formation to a degree that was equivalent to that obtained with sulindac, a potent nonsteroidal anti-inflammatory drug (36). It is important to point out that the prevailing belief of white tea being more effective than green tea is primarily founded on the higher EGCG content in white tea. As such, this advantage over green tea might be lost when the tea extracts are standardized to contain the same amount of EGCG. However, in our study, we found that

standardized WTE containing the same amounts of total catechin and EGCG levels as GTE from the same vendor were actually more potent in inducing apoptosis and augmenting 15-LOX-1, 15-LOX-2, and PPAR- $\gamma$  mRNA expression. What substances in the WTE that might actually be responsible for this phenomenon is unclear. Conceivably, there may be micronutrients or substances that have not yet been characterized and might be a subject of interest for future investigation. Our observations further support the potential advantage of WTE over GTE. This is of particular interests in view of the recent concern over emerging data showing the limited bioavailability of EGCG with green tea studies *in vivo* (5). Our findings provide important rationales to further investigate the anticarcinogenic properties of WTE and its potential for lung cancer chemoprevention.

## References

- Tijburg LB, Mattern T, Folts JD, Weisgerber UM, Katan MB. Tea flavonoids and cardiovascular disease: a review. *Crit Rev Food Sci Nutr* 1997;37:771–85.
- Dreosti IE. Antioxidant polyphenols in tea, cocoa, and wine. *Nutrition* 2000;16:692–4.
- Siddiqui IA, Adhami VM, Saleem M, Mukhtar H. Beneficial effects of tea and its polyphenols against prostate cancer. *Mol Nutr Food Res* 2006;50:130–43.
- Yuan JM, Gao YT, Yang CS, Yu MC. Urinary biomarkers of tea polyphenols and risk of colorectal cancer in the Shanghai Cohort Study. *Int J Cancer* 2007;120:1344–50.
- Yang CS, Sang S, Lambert JD, Hou Z, Ju J, Lu G. Possible mechanisms of the cancer-preventive activities of green tea. *Mol Nutr Food Res* 2006;50:170–5.
- Dashwood RH. Early detection and prevention of colorectal cancer. *Oncol Rep* 1999;6:277–81.
- Ahmed S, Rahman A, Hasnain A, Lalonde M, Goldberg VM, Haqqi TM. Green tea polyphenol epigallocatechin-3-gallate inhibits the IL-1 $\beta$ -induced activity and expression of cyclooxygenase-2 and nitric oxide synthase-2 in human chondrocytes. *Free Radic Biol Med* 2002;33:1097–105.
- Metz N, Lobstein A, Schneider Y, et al. Suppression of azoxymethane-induced preneoplastic lesions and inhibition of cyclooxygenase-2 activity in the colonic mucosa of rats drinking a crude green tea extract. *Nutr Cancer* 2000;38:60–4.
- Luceri C, Caderni G, Sanna A, Dolara P. Red wine and black tea polyphenols modulate the expression of cyclooxygenase-2, inducible nitric oxide synthase and glutathione-related enzymes in azoxymethane-induced F344 rat colon tumors. *J Nutr* 2002;132:1376–9.
- Lu G, Liao J, Yang G, Reuhl KR, Hao X, Yang CS. Inhibition of adenoma progression to adenocarcinoma in a 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis model in A/J mice by tea polyphenols and caffeine. *Cancer Res* 2006;66:11494–501.
- Record IR, Dreosti IE. Protection by tea against UV-A + B-induced skin cancers in hairless mice. *Nutr Cancer* 1998;32:71–5.
- Sato D. Inhibition of urinary bladder tumors induced by *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine in rats by green tea. *Int J Urol* 1999;6:93–9.
- de Boer JG, Yang H, Holcroft J, Skov K. Chemoprotection against *N*-nitrosomethylbenzylamine-induced mutation in the rat esophagus. *Nutr Cancer* 2004;50:168–73.
- Santana-Rios G, Orner GA, Xu M, Izquierdo-Pulido M, Dashwood RH. Inhibition by white tea of 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine-induced colonic aberrant crypts in the F344 rat. *Nutr Cancer* 2001;41:98–103.
- Chung FL, Morse MA, Eklind KI, Xu Y. Inhibition of tobacco-specific nitrosamine-induced lung tumorigenesis by compounds derived from cruciferous vegetables and green tea. *Ann N Y Acad Sci* 1993;686:186–201.
- Santana-Rios G, Orner GA, Amantana A, Provost C, Wu SY, Dashwood RH. Potent antimutagenic activity of white tea in comparison with green tea in the Salmonella assay. *Mutat Res* 2001;495:61–74.
- Ara G, Teicher BA. Cyclooxygenase and lipoxygenase inhibitors in cancer therapy. *Prostaglandins Leukot Essent Fatty Acids* 1996;54:3–16.
- Mao JT, Cui X, Reckamp K, et al. Chemoprevention strategies with cyclooxygenase-2 inhibitors for lung cancer. *Clin Lung Cancer* 2005;7:30–9.
- Shankaranarayanan P, Nigam S. IL-4 induces apoptosis in A549 lung adenocarcinoma cells: evidence for the pivotal role of 15-hydroxyeicosatetraenoic acid binding to activated peroxisome proliferator-activated receptor  $\gamma$  transcription factor. *J Immunol* 2003;170:887–94.
- Shappell SB, Gupta RA, Manning S, et al. 15S-Hydroxyeicosatetraenoic acid activates peroxisome proliferator-activated receptor  $\gamma$  and inhibits proliferation in PC3 prostate carcinoma cells. *Cancer Res* 2001;61:497–503.
- Dashwood WM, Orner GA, Dashwood RH. Inhibition of  $\beta$ -catenin/Tcf activity by white tea, green tea, and epigallocatechin-3-gallate (EGCG): minor contribution of H<sub>2</sub>O<sub>2</sub> at physiologically relevant EGCG concentrations. *Biochem Biophys Res Commun* 2002;296:584–8.
- Sartippour M, Heber D, Lu Q, Ma M, Go VL, Nguyen M. Green tea inhibits breast cancer growth and angiogenesis. *Nut Cancer* 2001;40:149–56.
- Chow HH, Hakim IA, Vining DR, et al. Effects of dosing condition on the oral bioavailability of green tea catechins after single-dose administration of Polyphenon E in healthy individuals. *Clin Cancer Res* 2005;11:4627–33.
- Mao JT, Tsu IH, Dubinett SM, et al. Modulation of pulmonary leukotriene B<sub>4</sub> production by cyclooxygenase-2 inhibitors and lipopolysaccharide. *Clin Cancer Res* 2004;10:6872–8.
- Kersten S, Desvergne B, Wahli W. Roles of PPARs in health and disease. *Nature* 2000;405:421.
- Satoh T, Toyoda M, Hoshino H, et al. Activation of peroxisome proliferator-activated receptor- $\gamma$  stimulates the growth arrest and DNA-damage inducible 153 gene in non-small cell lung carcinoma. *Oncogene* 2002;21:2171–80.
- Chen GG, Xu H, Lee JF, et al. 15-hydroxy-eicosatetraenoic acid arrests growth of colorectal cancer cells via a peroxisome proliferator-activated receptor  $\gamma$ -dependent pathway. *Int J Cancer* 2003;107:837–43.
- Hsi LC, Wilson L, Nixon J, Eling TE. 15-lipoxygenase-1 metabolites down-regulate peroxisome proliferator-activated receptor  $\gamma$  via the MAPK signaling pathway. *J Biol Chem* 2001;276:34545–52.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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29. Shureiqi I, Jiang W, Zuo X, et al. The 15-lipoxygenase-1 product 13-S-hydroxyoctadecadienoic acid down-regulates PPAR- $\delta$  to induce apoptosis in colorectal cancer cells. *Proc Natl Acad Sci U S A* 2003;100:9968–73.
30. Lai CK, Phillips GD, Jenkins JR, Holgate ST. The effect of inhaled 15-(s)-hydroxyeicosatetraenoic acid (15-HETE) on airway calibre and non-specific responsiveness in normal and asthmatic human subjects. *Eur Respir J* 1990;3:38–45.
31. Moreno JJ. New aspects of the role of hydroxyeicosatetraenoic acids in cell growth and cancer development. *Biochem Pharmacol* 2009;77:1–10.
32. Chang TH, Szabo E. Induction of differentiation and apoptosis by ligands of peroxisome proliferator-activated receptor  $\gamma$  in non-small cell lung cancer. *Cancer Res* 2000;60:1129–38.
33. Jin YH, Yoo KJ, Lee YH, Lee SK. Caspase 3-mediated cleavage of p21WAF1/CIP1 associated with the cyclin A-cyclin-dependent kinase 2 complex is a prerequisite for apoptosis in SK-HEP-1 cells. *J Biol Chem* 2000;275:30256–63.
34. Nicholson DW, Ali A, Thornberry NA, et al. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 1995;376:37–43.
35. Muto S, Yokoi T, Gondo Y, et al. Inhibition of benzo[a]pyrene-induced mutagenesis by (-)-epigallocatechin gallate in the lung of rpsL transgenic mice. *Carcinogenesis* 1999;20:421–4.
36. Orner GA, Dashwood WM, Blum CA, Diaz GD, Li Q, Dashwood RH. Suppression of tumorigenesis in the Apc(min) mouse: down-regulation of  $\beta$ -catenin signaling by a combination of tea plus sulindac. *Carcinogenesis* 2003;24:263–7.
37. Bishop-Bailey D, Hla T. Endothelial cell apoptosis induced by the peroxisome proliferator-activated receptor (PPAR) ligand 15-deoxy- $\Delta$ 12, 14-prostaglandin J2. *J Biol Chem* 1999;274:17042–8.
38. Hashimoto K, Ethridge RT, Saito H, Rajaraman S, Evers BM. The PPAR $\gamma$  ligand, 15d-PGJ2, attenuates the severity of cerulein-induced acute pancreatitis. *Pancreas* 2003;27:58–66.

# Cancer Prevention Research

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