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Green tea inhibits cycolooxygenase-2 in non-small cell lung cancer cells through the induction of Annexin-1

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ABSTRACT

Elevated cyclooygenase-2 (COX-2) expression is frequently observed in human non-small cell lung cancer (NSCLC) and associated with poor prognosis, indicating critical involvement of the inflammatory pathway in lung carcinogenesis. Recently, we found that green tea extract (GTE) induced Annexin-1 (ANX1) in the lung adenocarcinoma A549 cells. ANX1 is a glucocorticoid-inducible 37 kDa protein involved in a wide range biological function and is an important anti-inflammatory mediator. The present study further examines the interplay between the expressions and production of ANX1, COX-2, phospholipase A_2 ($cPLA_2$) and prostaglandin E_2 (PGE_2) following the treatment of NSCLC cell lines with GTE. We found that GTE induced ANX1 and inhibited COX-2 expression in lung cancer A549, H157 and H460 cell lines. Addition of pro-inflammatory cytokine IL-1β diminished GTE-induced ANX1. Silence of ANX1 in cells abrogates the inhibitory activity on COX-2, indicating that the anti-inflammatory activity of GTE is mediated at least partially by the up-regulation of ANX1. However, differential pattern of inhibitory effects of ANX1 on $cPLA_2$ expression was observed among various cell types, suggesting that the anti-inflammatory activity mediated by ANX1 is cell type specific. Our study may provide a new mechanism of GTE on the prevention of lung cancer and other diseases related to inflammation.

1. Introduction

Lung cancer is the leading cause of cancer-related death in the United States [1]. Recently it is increasingly recognized that inflammation contributes to the development of lung cancer. An important component of the inflammatory pathways is cyclooxygenase-2 (COX-2)/prostaglandin E_2 (PGE_2). Increased COX-2 expression is often observed in human non-small cell lung cancer (NSCLC). It is predominantly responsible for the overproduction of PGE_2, which is associated with a variety of well-established carcinogenic mechanisms, such as resistance to apoptosis, increased angiogenesis, suppression of host immunity, and enhancement of invasion and metastasis [2]. Therefore, COX-2 is considered an important molecular target for lung cancer therapy and chemoprevention.

Annexins are a family of structurally related proteins that exhibit calcium dependent binding to anionic phospholipids. Annexin-1 (ANX1) is the first of its 13 members in the family and was originally identified as a glucocorticoid-inducible 37 kDa protein expressed in epithelial cells [3]. It is a phospholipase A2 inhibitor and is involved in a wide range of biological functions including cell differentiation, cell growth arrest, anti-inflammation and apoptosis induction [4–7]. ANX1 has been studied extensively for its roles in human NSCLC cell line A549. Croxtall et al. found that the dexamethasone increased ANX1 synthesis in A549 cells, which in turn inhibited PGE_2 production and cell growth [5,8]. ANX1 gene deletion in mice leads to up-regulation of expression of COX-2 and $cPLA_2$ in lung and some other tissues and exhibit an exaggerated response to the inflammatory stimuli characterized by an increase in leukocyte emigration and IL-1β generation. These mice also exhibit a partial or complete resistance to the anti-inflammatory effects of glucocorticoids compared with that of wild-type control [9]. ANX1 in the regulation by these steroids were demonstrated in varies human diseases such as acute and chronic inflammation, ischaemic damage, pain and fever [10]. These and other experimental models provided strong evidence that ANX1 is involved in the regulation of inflammation as well as other signaling pathways.

Green tea (*Camellia sinensis* leaves) contains polyphenols that are naturally occurring antioxidants and is a promising chemopreventive agent [11]. Laboratory and animal studies have shown a

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protective effect of green tea against a variety of cancer, including lung cancer. For example, green tea has been shown to inhibit 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced tumorigenicity in A/J mice [12]. However, the antineoplastic mechanism of green tea in lung cancer is not fully understood. We recently identified a functional protein target ANX1 induced by GTE in human urothelial MC-T11 cells and A549 cells using proteomic approach [13,14]. We found that GTE-induced ANX1 up-regulation in A549 cells is dose-dependent and occurs at the transcriptional level. Further, GTE-induced ANX1 expression appears to mediate cytoskeletal actin remodeling. ANX1 up-regulation stimulates actin polymerization, which in turn results in the increase of cell adhesion and decrease of motility in these cell lines. The current study examined the GTE-induced expressions of ANX1 and the inhibition of COX-2, $cPLA_2$ and PGE_2 by GTE treatment in NSCLC cell lines. Our results show that GTE is effective in inducing ANX1 expression which in turn inhibits COX-2 expression and PGE_2 production in NSCLC cell lines. Our findings indicate that the anti-inflammatory activity of GTE is mediated at least partially by GTE-induced ANX1.

2. Materials and methods

2.1. Materials

GTE was obtained from Pharmanex Inc. (Provo, UT, USA). The purity of the catechins in the GTE was 84% [14]. The GTE is a mixture of many catechin compounds, consisted of epigallocatechin gallate (EGCG, 43.0% by weight), epicatechin-3-gallate (ECG, 13.7%), epicatechin (EC, 6.0%), gallocatechin gallate (GCG, 5.6%), epigallocatechin (EGC, 4.0%). The GTE contained less than 0.3% caffeine.

2.2. Cell culture and GTE stimulation

Lung cancer A549 (ATCC, Manassas, VA, USA), NCI-H460 and NCI-H157 cell lines (National Cancer Institute, Bethesda, MD, USA) were grown in 90% RPMI 1640 medium (Mediatech Inc., Herndon, VA, USA) with 1% penicillin and streptomycin mix solution (Invitrogen, Carlsbad, CA, USA) and 10% fetal bovine serum (FBS). Non-neoplastic bronchial epithelial cell line BEAS-2B cell line (ATCC) was maintained in LHC-9 medium (Invitrogen). Cultures were maintained at 37 °C in 5% CO_2 and 95% air. Logarithmically growing cells were harvested and seeded at an initial density of 1×10^6 cells in 5 mL of fresh medium in 60-mm Petri dishes. After overnight proliferation, the adherent cells were treated with GTE at the final concentrations of 0, 10, 20, and 40 μg/mL. Cells were harvested after 24 h. Stock solution of IL-1β (BD Biosciences, San Diego, CA, USA) was prepared in BSA at the concentration of 1000 ng/mL. It was added to the cell culture medium at the final concentration of 1 ng/mL for 16 h, followed by the addition of GTE.

Cell proliferation was determined at 24, 48, and 72 h points. H157 and H460 cells were plated in 96-well plates (0.5×10^4 cells/well) and treated with GTE at 0–1000 μg/mL concentrations. Viable cells were determined using the Cell Proliferation Assay kit (Chemicon, Temecula, CA, USA) according to the manufacturer's instructions. To evaluate the cytotoxicity of GTE, the same assay was performed with cells seeded in 96-well plates at a density of 1.0×10^4 cells/well according to the manufacturer's recommendation and treated with GTE at 0, 1.25, 2.5, 5 10, 20, 40, 80, 160, 320 and 640 μg/mL concentration at 37 °C for 24 h. The IC_{50} was calculated from the 24 h viability data based on the OD reading as previously described [15]. All treatments were performed in triplicate.

2.3. Immunoblot analysis

Immunoblot analyses were performed as previously described [15]. Reactions with the primary antibody (1:5000, BD), COX-2 (1: 500, Cayman Chemical, Ann Arbor, MI, USA), $cPLA_2$ (1:500 Santa Cruz Biotechnology, Santa Cruz, CA, USA), or β-actin (1:500, Sigma, St. Louis, MO, USA) in TBST buffer containing 3% dry milk were carried out at 4 °C overnight. After extensive washing, membranes were placed on a shaker with biotinylated secondary IgG for 1 h. Upon further washing, membranes were reacted with ECL detection reagents (Amersham Biosciences, Piscataway, NJ, USA) immediately prior to autoradiography. The relative levels of ANX1 protein were determined by scanning densitometry using Alpha-Imager 2000 software (Alpha Innotech, Cannock, UK).

2.4. Immunofluorescence analysis

Immunofluorescence analysis was performed as previously described [14]. Cells cultured directly on 1 cm diameter cover glass were fixed with 3.7% paraformaldehyde for 0.5 h were incubated with 1:3000 mouse monoclonal anti-annexin-I (BD) for 1 h, 1:500 Cy3-conjugated AffiniPure Goat Anti-Mouse IgG (HþL) (Jackson ImmunoResearch Lab, West Grove, PA, USA) for 0.5 h. Images were generated using a Nikon TE300 microscope equipped with an Imaging Microimager II digital camera. ANX1 fluorescence intensity was analyzed using NIH Image-J software.

2.5. ANX1 siRNA transfection

Three small interfering RNA (siRNA)-coding oligos against human ANX1 mRNA were purchased from (Qiagen Inc., Valencia, CA, USA) as previously described [14]. The siRNA transfection of cells was performed using the transfection reagent (Qiagen) according to the manufacturer's instructions.

2.6. PGE_2 measurements

Cells were stimulated with IL-1β (1 ng/mL) for 16 h and then treated with GTE. PGE_2 concentration in each treatment group (with or without IL-1β stimulation) was measured by enzyme immunoassay using a PGE_2 enzyme immunoassay kit (Cayman) according to the manufacturer's instructions. All measurements were made in triplicates for each treatment group. Experiments were performed in triplicate.

2.7. RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted from cells using a TRIzol reagent (Life Technologies, Grand Island, NY, USA), and purified using the RNeasy Mini Kit (Qiagen) as previously described [14]. The following PCR amplification parameters were used: 5 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Data acquisition was set at the chain extension step and the melt curve data collection analysis was performed between 55 and 95 °C, with 0.5 °C-increments. All data were analyzed using the iCycler IQ optical system software (BioRad).

2.8. Statistical analysis

Descriptive statistics, such as mean and SD, were used to summarize the results. Data were analyzed by paired student *t*-test or ANOVA. Statistical significance was defined by *p*-value of 0.05.

3. Results

3.1. Inhibition of cell growth by GTE

We first examined the IC_{50} and time-, as well as dose-effect of GTE on proliferation of H157 and H460 lung cancer cells. We previously reported that GTE inhibited A549 cell proliferation time- and dose-dependently with IC_{50} being 112 μg/mL [15]. We showed that GTE time- and dose-dependently inhibited H157 cells (Fig. 1) and H460 cells (data not shown) at 24, 48 and 72 h. The IC_{50} of GTE on H157 cells is 137 and on H460 cells is 111 μg/mL.

3.2. Effects of GTE on ANX1 expression in NSCLC cells

We previously reported that GTE dose-dependently increased ANX1 mRNA expression and protein production in A549 cells. We also showed dose-dependent increase of ANX1 protein production in H460 and H157 cells [14]. In this study we examined ANX1 production in H460 and H157 cells using immunofluorescence and RT-PCR analyses. Results from immunofluorescence analysis indicate that GTE dose-dependently increases ANX1 production in H157 cells (Fig. 2A top panel). In H460 cells, the GTE mediated increase in ANX1 was higher with lower doses of GTE (Fig. 2A, lower panel). We further examined ANX1 mRNA expression using quantitative RT-PCR analysis. In both H157 and H460 cell lines, GTE dose-dependently increased ANX1 mRNA expression (Fig. 2B). The difference of expression between Fig. 2A and B (mRNA versus protein) may be simply due to the different molecular levels examined. The induction of ANX1 by GTE was abrogated by ANX1-targeted siRNA transfection into the cell whereas ANX1 non-targeted siRNA did not block the induction of ANX1 by GTE.

3.3. Inhibition of COX-2, $cPLA_2$ in NSCLC cells by GTE-induced ANX1

We then examined the concomitant expression of ANX1, COX-2, and $cPLA_2$ in the presence or absence of pro-inflammatory cytokine IL-1β by immunoblot analysis. In A549 and H460 cells, the dose-dependent increase in ANX1 induced by GTE was accompanied by decreases in both the constitutive expressions of COX-2 and $cPLA_2$ (Fig. 3A). As expected, IL-1β stimulation increased the expression of COX-2, it concomitantly decreased the expression of ANX1. Although IL-1β increased COX-2 expression the dose-responsive suppression of COX-2 and $cPLA_2$ by GTE was still apparent. The stimulation of A549 cells with IL-1β results in the up-regulation of COX-2 expression and activation of $cPLA_2$, but down-regulation of the expression of ANX1 was reported previously [16].

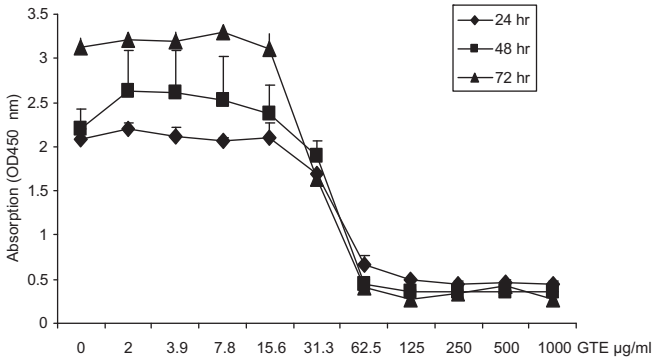

Fig. 1. Dose-dependant effect of GTE on cell proliferation in H157 cells. Cells were plated at a density of 0.5×10^4 cells/mL, and cultured at various times (24, 48, and 72 h) in medium containing GTE at various concentrations as indicated. Cell viability was assayed using the MTT method. Untreated cells served as controls. Values represent the mean ± SD of three different experiments.

To determine if the GTE induced ANX1 contributed to the decrease in COX-2 and $cPLA_2$ production, we transfected A549 cells with ANX1-specific siRNA. Silencing of ANX1 blocked the decrease in COX-2 and $cPLA_2$ production by GTE (Fig. 3B). Non-specific siRNA produced dose-responsive decrease in COX-2 protein level with 13% suppression at 40 μg/mL GTE dose (lane 8 versus lane 5 from the left) based on the densitometry. At 20 μg/mL GTE dose, the range of difference in COX-2 level is collectively 28% (lane 7 versus lane 3 from the left), and 23% when adjusted to non-specific-siRNA levels alone (lane 5 from the left). In H157 cells transfected with ANX1 non-specific siRNA, GTE treatment led to a reduction of COX-2 production, but not $cPLA_2$ (Fig. 3C). Transfection with ANX1 siRNA demonstrated that the reduction of COX-2 was mediated by GTE induced ANX1 production. This was also observed in H460 cells transfected with siRNA (data not shown). It is important to note that siRNA blocked the ANX1 production following the GTE treatment, but the basal production of the protein remained unaffected and highly expressed during this time. This is compatible with the dexamethasone-induced ANX1 expression in A549 cells transfected with an antisense oligonucleotide derived from ANX1 cDNA, in which the newly-induced protein was inhibited but pre-existing protein remained unaffected [8].

We also examined the protein expressions of ANX1 and the inhibition of COX-2 by GTE treatment in the non-neoplastic bronchial epithelial cell line BEAS-2B. Our results show that GTE does not change the expressions ANX1, COX-2 and $cPLA_2$ (data not shown).

GTE inhibited COX-2 mRNA expression in both H157 (Fig. 3D) and H460 (Fig. 3E) cell lines and GTE induced-ANX1 contributed to the reduction. GTE treatment significantly reduced the COX-2 expression in H460 cells and in cells transfected with non-targeted siRNA, whereas silencing of ANX1 with siRNA partially abrogated the reduction of COX-2 mRNA levels by GTE.

3.4. Inhibition of PGE_2 in NSCLC cells by GTE

Up-regulation of COX-2 by inflammatory cytokine such as IL-1β is responsible for the over production of PGE_2. Over production of PGE_2 in turn mediates a variety of carcinogenic mechanism such as increase cellular proliferation. We further determined the effects of GTE on PGE_2 production in A549 cells. GTE dose-dependently inhibited the constitutive and IL-1β stimulated PGE_2 production (Fig. 4A). In presence of IL-1β GTE treatment of the H157 cells transfected with non-specific siRNA reduced the level of PGE_2. In ANX1-silenced cells, however, levels of PGE_2 remain nearly unchanged upon treatment of GTE (Fig. 4B). A similar pattern is observed in H460 cells (data not shown), indicating that GTE-induced ANX1 is at least partly responsible for the inhibition of PGE_2.

4. Discussion

Our current study identifies the anti-inflammatory activity of GTE is, in part, mediated by the upregulation of ANX1 and down regulation of COX-2/PGE_2 in NSCLC cells. Our previous studies have shown that GTE increases protein expression of ANX1 in urothelial tumor MC-T11, lung cancer A549 [13,14] and pancreatic cancer HPAF-II (unpublished data) cell lines. The present study demonstrated that GTE also induces ANX1 mRNA expression and protein production in H460 (large cell lung carcinoma) and H157 (squamous cell carcinoma) cell lines. We further demonstrated that GTE down-regulates COX-2, $cPLA_2$ and PGE_2 expressions in NSCLC cell lines. At protein levels, IL-1β stimulation increased the expression of COX-2, it concomitantly decreased the expression of ANX1. Although IL-1β increased COX-2 expression, the dose-dependent suppression of COX-2 and $cPLA_2$ by GTE was still

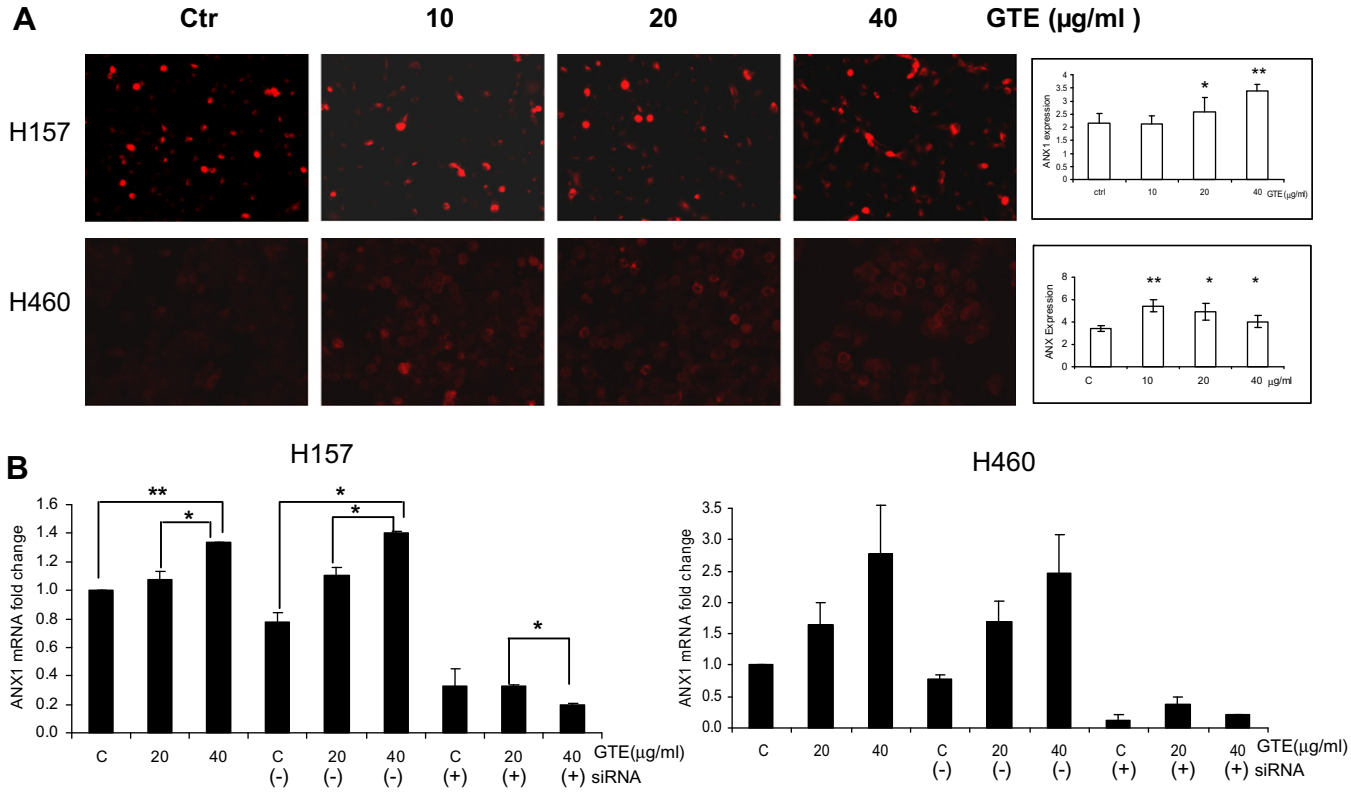

Fig. 2. Dose-dependent effect of GTE on ANX1 expressions in NSCLC H460 and H157 cell lines. (A) Quantitative immunofluorescence analysis of ANX1 in cells treated with 0 (C), 10, 20, or 40 μg/mL of GTE for 17 h. Cells were washed, fixed, and labeled for ANX1. Images were taken using a Nikon Eclipse E400 microscope at 20× object. (B) Quantitative real-time PCR analysis of ANX1 with and without ANX1-specific or non-specific siRNA interference on cells treated with same doses of GTE for 24 h under the conditions as specified. (−) – Negative control with ANX1non-specific siRNA transfection, (+) – AXN1-specific siRNA transfection. Note that AXN1-specific siRNA transfection blocked the GTE-induced AXN1 production whereas non-specific siRNA had no such effects. Triplicate determinations were averaged at each data-point. Each experiment was performed in duplicate. Data represents one of the two experiments.

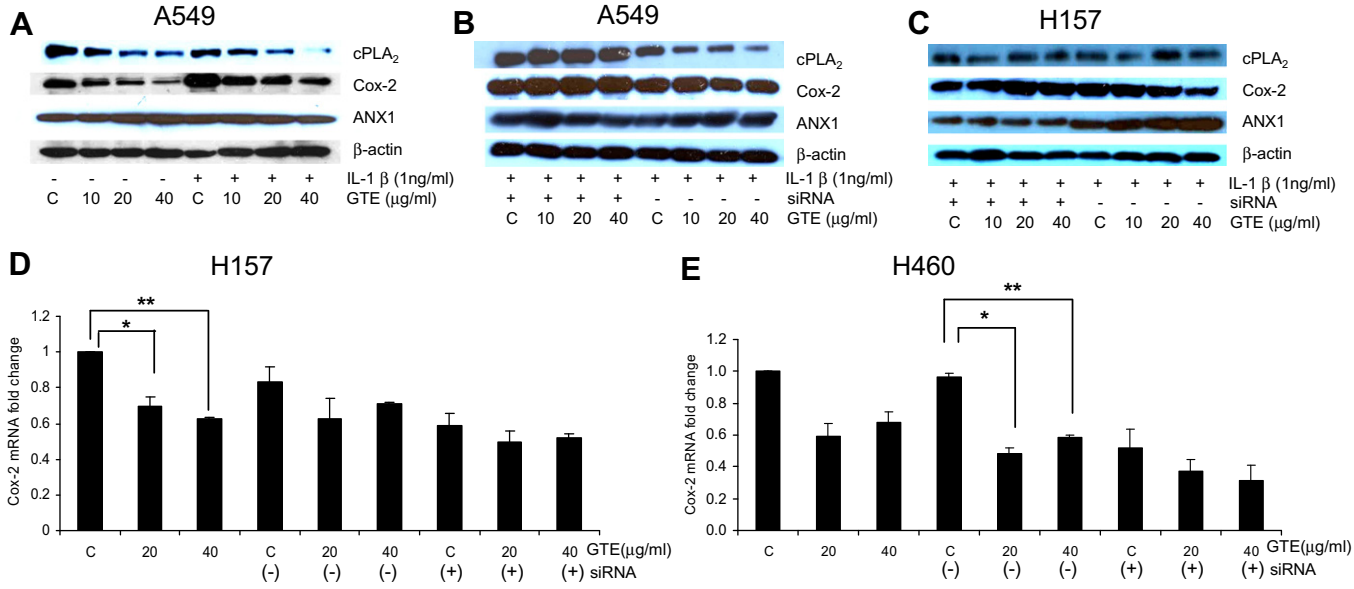

Fig. 3. Dose-dependent effect of GTE on the expression of ANX1, COX-2, and $cPLA_2$ in NSCLC cell lines. (A) Western blot analysis of GTE on ANX1, COX-2, $cPLA_2$ expressions with or without IL-1β stimulation A549 cells; Western blot analysis of GTE on ANX1, COX-2, $cPLA_2$ expressions in A549 (B) and H157 (C) cells transfected with siRNA in the presence of IL-1β; and quantitative real-time PCR analysis of ANX1 siRNA interference on the change of COX-2 in H460 (D) and H157 (E) cells. Cells treated with 0 (C), 20, or 40 μg/mL of GTE for 24 h under the conditions as specified. (−) – Negative control with ANX1 non-specific siRNA transfection, (+) – AXN1-specific siRNA transfection. Triplicate determinations were averaged at each data-point. Each experiment was performed in triplicate. Data represents one of the three experiments.

apparent. These data are consistent with a previous study on the regulation of COX-2 and $cPLA_2$ by dexamethasone induced ANX1 following IL-1β stimulation of A549 cells [16]. Silencing of ANX1 with ANX1-specific siRNA blocked the decrease in COX-2, $cPLA_2$

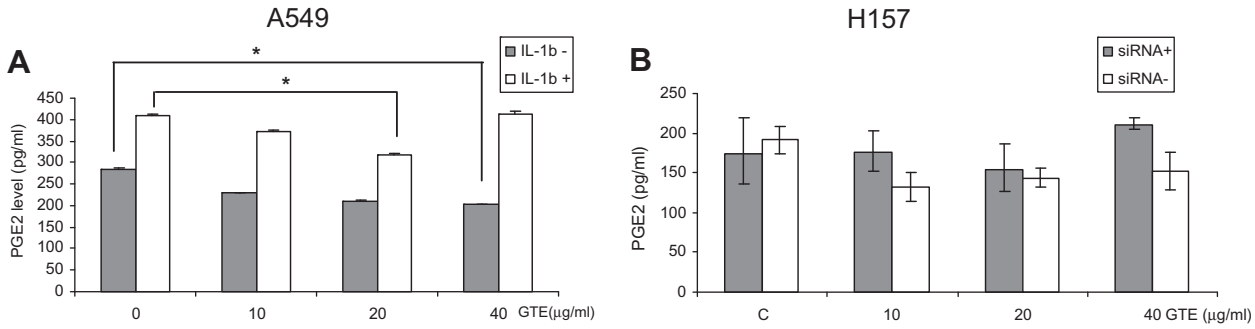

Fig. 4. Dose-dependant effect of GTE on PGE_2 production in NSCLC cell lines. (A) Inhibition of PGE_2 in A549 cells by GTE with or without IL-1β stimulation. (B) Effect of ANX1-specific siRNA interference on PGE_2 in H157 cells pretreated with IL-1β. ANX1 non-specific siRNA (−) served as another level of control. PGE_2 was measured by Elisa method. Triplicate determinations were averaged at each data-point. Each experiment was performed in duplicate. Data represents the average of the three experiments.

and PGE_2 production by GTE, indicating that the suppression of COX-2 and $cPLA_2$ by GTE is mediated via the up-regulation of ANX1.

Not surprisingly, ANX1-specific siRNA transfection did not completely abrogate the inhibition of COX-2 and $cPLA_2$ by GTE. This is likely due to the fact that GTE and its major polyphenol constituents target multiple inflammatory pathways through the modulations of NF-κB, EGFR/HER2 and mitogen-activated protein kinase pathways [17,18].

The involvement of glucocorticoid induced-ANX1 in the inhibition of PGE_2 was previously reported on the A549 cells. Croxtall et al. found that the dexamethasone increased ANX1 synthesis in A549 cells, which in turn inhibited PGE_2 production and cell growth. The addition of glucocorticoid to A549 cells results in ANX1 translocation to the membrane compartment and subsequent externalization which inhibited prostaglandin release by affecting $cPLA_2$ activation through an effect of EGF signaling, thereby blocking cell proliferation [5,19]. Green tea and its polyphenol constituents have been reported to inhibit PGE_2 production [20,21]. Koeberle et al. reported that EGCG, a major constituent of green tea up to 30 μM suppressed PGE_2 production through inhibiting the transformation of PGH_2 to PGE_2 catalyzed by microsomal PGE_2 synthase-1 (mPGES-1, IC_{50} = 1.8 μM) [21]. Moon et al. reported that EGCG at the concentration of 25 μM inhibited COX-2 but increased PGE_2 production and mPGES-1 expression in A549 cells. IL-1β stimulated mPGES-1 which was further induced by EGCG. In comparable concentrations, EGC showed minimal induction of mPGES-1 while EC inhibited mPGES-1 [22]. Our data is consistent with these reports. In A549 cells, lower doses of GTE inhibited both the constitutive and IL-1β stimulated production of PGE_2. However, at high concentration, GTE (40 μg/mL contains 37.6 μM EGCG) failed to inhibit PGE_2 (Fig. 4A). In 157 cell line, the inhibition of PGE_2 by GTE in non-targeted cells is partially reversed in ANX1 targeted cells.

The association between chronic inflammation and lung cancer has been extensively reported. Aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs), has been reported to reduce a variety of malignancies, including lung cancer. The best known targets of NSAIDs are COX enzymes, which convert arachidonic acid to prostaglandins (PGs) and thromboxane. COX-2 derived PGE_2 can promote tumor growth by binding its receptors and activating signaling pathways which control cell proliferation, migration, apoptosis, and/or angiogenesis [23]. One recent study revealed the modified nonsteroidal anti-inflammatory drugs, nitric oxide-donating aspirin and phospho-aspirin, but not conventional NSAID, induced ANX1 in cultured human colon and pancreatic cancer cells. It was found that the induction of ANX1 by glucocorticoids was proportional to their anti-inflammatory potency, as was the suppression of NF-κB activity, which was accompanied by enhanced apoptosis and inhibition of cell growth mediated by changes in NF-κB-dependent cell signaling [24].

Although regular use of aspirin and other NSAIDs is associated with reduce risk of developing lung cancer in animal models [25] and in smokers [26], the use of various NSAIDs other than aspirin is associated with higher cardiovascular risk [27]. Thus it is necessary to develop more effective chemopreventive agents that target the COX-2/PGE_2 pathways with minimal toxicity [23]. Tea has been a popular drink worldwide and generally considered as a safe food item.

To summarize, our studies demonstrate that GTE is effective in inducing ANX1 expression in a variety of NSCLC cell lines, which resulted in the inhibition of COX-2 expression. The anti-inflammatory activity of GTE induced-ANX1 was most noticeable in adenocarcinoma A549 cell line, as evidenced by the simultaneous inhibition of COX-2, $cPLA_2$ and PGE_2. Silence of ANX1 in cells abrogated the inhibitory activity of GTE on COX-2 and PGE_2, indicating that the anti-inflammatory activity of GTE is mediated at least partially by the up-regulation of ANX1. However, differential pattern of inhibitory effects of ANX1 on $cPLA_2$ expression was observed among various cell types, suggesting that the anti-inflammatory activity mediated by ANX1 is cell type specific. In H157 and H460 cells, ANX1 is responsible for the inhibition of COX-2 at the protein levels and PGE_2, but not for the $cPLA_2$ level. Our study may provide an additional mechanism of GTE on the prevention of lung cancer and other diseases related to inflammation.

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