Thoracic Cancer ISSN 1759-7706

ORIGINAL ARTICLE

Novel link between prostaglandin E_2 (PGE_2) and cholinergic signaling in lung cancer: The role of c-Jun in PGE_2-induced α7 nicotinic acetylcholine receptor expression and tumor cell proliferation

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Keywords
α7 nAChR; c-Jun; EP4; lung cancer; PGE_2.

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Received: 16 October 2014;
Accepted: 24 November 2014.

doi: 10.1111/1759-7714.12219

Thoracic Cancer **6** (2015) 488–500

Abstract

Background: Cyclooxygenase-2-derived prostaglandin E_2 (PGE_2) stimulates tumor cell growth and progression. α7 nicotinic acetylcholine receptor (nAChR) is a major mediator of cholinergic signaling in tumor cells. In the present study, we investigated the mechanisms by which PGE_2 increases non-small cell lung cancer (NSCLC) proliferation via α7 nAChR induction.

Methods: The effects of PGE_2 on α7 nAChR expression, promoter activity, and cell signaling pathways were detected by Western blot analysis, real time reverse transcriptase polymerase chain reaction, and transient transfection assay. The effect of PGE_2 on cell growth was determined by cell viability assay.

Results: We found that PGE_2 induced α7 nAChR expression and its promoter activity in NSCLC cells. The stimulatory role of PGE_2 on cell proliferation was attenuated by α7 nAChR small interfering ribonucleic acids (siRNA) or acetylcholinesterase. PGE_2-induced α7 nAChR expression was blocked by an antagonist of the PGE_2 receptor subtype EP4 and by EP4 siRNA. Furthermore, PGE_2 enhanced α7 nAChR expression via activation of c-Jun N-terminal kinase (JNK), phosphatidylinositol 3-kinase (PI3-K), and protein kinase A (PKA) pathways followed by increased c-Jun expression, a critical transcription factor. Blockade of c-Jun diminished the effects of PGE_2 on α7 nAChR promoter activity and protein expression, and cell growth.

Conclusion: Our results demonstrate that PGE_2 promotes NSCLC cell growth through increased α7 nAChR expression. This effect is dependent on EP4-mediated activation of JNK, PI3K, and PKA signals that induce c-Jun protein expression and α7 nAChR gene promoter activity. Our findings unveil a novel link between prostanoids and cholinergic signaling.

Introduction

Lung cancer is the leading cause of cancer-related mortality in the United States. Although multiple therapies have been developed during recent decades, non-small cell lung cancer (NSCLC), which accounts for the majority of lung cancer cases, still carries a five-year survival rate of 15%.[1] Therefore, it is vital to understand the molecular mechanisms underlying lung cancer growth and progression with the objective of identifying new targets for therapy.

Cyclooxygenase-2 (COX-2) is considered a target for the treatment of NSCLC, as overexpression of COX-2 has been found in a wide variety of human cancers, including lung cancer.[2,3] Elevated COX-2 expression also appears to portend shorter survival among patients with early stage NSCLC,[3] and high levels of COX-2 in tumor cells result in increased

proliferation and cell survival, and enhanced angiogenesis.[4] *In vivo* studies have demonstrated that high doses of the selective COX-2 inhibitor, celecoxib, significantly inhibit lung tumor growth.[5] However, the prolonged use of high dose celecoxib and other COX-2 inhibitors is associated with unacceptable cardiovascular side effects, which result from the inhibition of antithrombotic prostaglandin I_2 production.[6,7] Consequently, to identify safe and efficient agents for therapy, researchers are focusing their attention to targets downstream of COX-2.

COX-2 converts arachidonic acid to prostaglandins during prostanoid synthesis and its products include prostaglandin E_2 (PGE_2).[8] PGE_2 is the major bioactive prostaglandin produced by COX-2 in many human malignancies. This mitogenic prostanoid promotes tumor growth by binding to cell surface prostanoid receptors (also termed EP receptors) and activating signaling pathways that regulate cell proliferation, migration, apoptosis, and angiogenesis.[8,9] The importance of PGE_2 is highlighted by studies showing that inhibition of its synthesis suppresses lung tumorigenesis *in vivo*.[10] It has been shown that PGE_2 stimulates cell proliferation and promotes resistance to pharmacologically induced apoptosis in a c-Myc and miR-17-92-dependent manner.[11] However, the molecular mechanisms underlying the effects of PGE_2 in lung carcinoma cells remain largely unknown.

Recently, activated non-neuronal cholinergic signaling has been implicated in human cancers, including lung cancer.[12,13] Through the synthesis and secretion of acetylcholine (ACh), this system can induce growth via autocrine and paracrine effects. ACh is synthesized and secreted by lung cancer cells and interacts with neighboring cells to stimulate growth by binding to nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors.[12,13] The α7 nAChR has received significant attention lately because it appears to mediate nicotine-induced proliferation in cancer cells, both *in vitro* and *in vivo*.[14,15]

Herein, we explore the potential link between PGE_2-induced NSCLC cell proliferation and induction of cholinergic signaling by α7 nAChR. Our studies show that PGE_2 stimulates NSCLC cell proliferation through activation of α7 nAChR. In addition, PGE_2 enhances α7 nAChR expression via activation of c-Jun N-terminal kinase (JNK), phosphatidylinositol 3-kinase (PI3-K)/Akt, and protein kinase A (PKA) followed by increased expression of c-Jun, a transcription factor that appears critical for α7 nAChR gene transcription. To our knowledge, this is the first published demonstration of a link between PGE_2 and cholinergic signaling via nAChRs in human lung carcinoma cells. Our findings unveil a novel molecular mechanism by which PGE_2 stimulates NSCLC cell growth and suggest potential molecular targets for the development of therapies against NSCLC.

Experimental procedures

Cell culture and chemicals

Human NSCLC cell lines (adenocarcinoma cell lines H1792, H1838, A549, and bronchioalveolar carcinoma cell line H358) were obtained from the American Type Culture Collection (Manassas, VA, USA) and routinely grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 50 IU/ml penicillin/streptomycin, and 1 μg amphotericin (complete medium), as previously described.[16] The CellTiter-Glo Luminescent Cell Viability Assay kit and the Dual-Luciferase Reporter Assay kit were obtained from Promega (Madison, WI, USA). Cayman Chemical Co. (Ann Arbor, MI, USA) provided 16, 16 dimethy-prostaglandin E_2 ($dmPGE_2$), the EP4 antagonist AH23848, and polyclonal antibody against EP4. The mitogen-activated protein kinase (MAPK) specific inhibitor, PD98059; the PI3-K inhibitor, LY294002, and Wortmannin; the PKA inhibitor, H-89; and polyclonal antibodies against SAPK/JNK and phospho-SAPK/JNK (THr183/Tyr185) were purchased from Cell Signaling (Beverly, MA, USA). The JNK inhibitor II was purchased from CalBiochem (San Diego, CA, USA). Polyclonal antibody against c-Jun was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Polyclonal antibody against α7 nAChR was purchased from Abcam Inc. (Cambridge, MA, USA). Acetylcholinesterase (AChE), antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Western blot analysis

The procedure was performed as previously described.[17] Protein concentrations were determined by the Bio-Rad protein assay (Hercules, CA, USA). Equal amounts of protein from whole cell lysates were solubilized in 2x sodium dodecyl sulfate (SDS)-sample buffer and separated on SDS (10%) polyacrylamide gels. Blots were incubated with antibodies against α7 nAChR, EP4, and c-Jun. After washing, the blots were incubated with a secondary goat antibody raised against rabbit immunoglobin (IgG) conjugated to horseradish peroxidase (1:2000–5000, Santa Cruz Biotechnology Inc.). The blots were washed, transferred to freshly made enhanced chemiluminescence (ECL) solution (Amersham, Arlington, IL, USA) for one minute, and exposed to X-ray film. GAPDH was used as loading control.

Real time reverse transcription polymerase chain reaction

Total ribonucleic acid (RNA) was isolated from the cells exposed to $dmPGE_2$ using RNA-Bee RNA isolation reagent (AMS Biotechnology, Abingdon, Oxfordshire, UK) according to the manufacturer's instructions. Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed using PerfeCTa SYBR Green FastMix (Quanta Biosciences, Gaithersburg, MD, USA). To amplify the α7 nAChR and GAPDH cDNA fragments, the sequences of PCR primers (sigma) were 5′-GGAGGAGGTCCGCTACATTG-3′ (forward) and 5′-TTGGGAGCCGACATCAGG-3′ (reverse) for α7 nAChR and 5′-ATGGGGAAGGTGAAGGTCG-3′ (forward) and 5′-CCATGTAGTTGAGGTCAATGAAGG-3′ (reverse) for GAPDH. The samples were processed using a Smart Cycler (Cepheid, Sunnyvale, CA, USA): denatured at 95°C for 120 seconds, followed by 40 cycles, each with temperature variations as follows: 95°C for one second, 60°C for 30 seconds. Results of the log-linear phase of the growth curve were analyzed and relative quantification was performed using the $2^{-\Delta\Delta CT}$ method with GAPDH as a housekeeping gene.

Cell viability assay

H1838 and H1792 cells were plated at the indicated densities (5000 cells/well) in 96-well multiwell culture plates (Costar, Cambridge, MA, USA). Cells were treated with AChE (50 U/mL) or AH23848 (5 μM) for two hours before exposure to $dmPGE_2$ (0.1 μM, 1 μM) for three to five days. Cell proliferation was evaluated using the CellTiter-Glo Luminescent Cell Viability Assay (Promega), a homogenous method of determining the number of viable cells in culture based on quantitation of the adenosine triphosphate (ATP) present, which signals the presence of metabolically active cells.

Treatment with α7 nicotinic acetylcholine receptors (nAChR), EP4 and c-Jun small interfering ribonucleic acid

The α7 nAChR, EP4, c-Jun small interfering ribonucleic acids (siRNAs), and control siRNA were purchased from Santa Cruz Biotechnology Inc. For the transfection procedure, cells were grown to 60% confluence, and α7 nAChR, EP4, c-Jun siRNAs, and control siRNA were transfected using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, the Lipofectamine 2000 reagent was incubated with serum-free medium for five minutes. Subsequently, the respective diluted siRNA was added. After incubation for 20 minutes at room temperature, the oligomer-Lipofectamine 2000 complexes were added to each well. The final concentration of siRNAs in each well was 100 nM. After culturing for 30 hours, cells were washed and resuspended in new culture media in the presence or absence of $dmPGE_2$ for additional time ranges for Western Blot and cell growth.

Site-directed mutagenesis

To prepare the site-directed mutant of the mouse α7 nAChR gene promoter, the following complimentary oligonucleotide primers were synthesized: mutated c-Jun (-455/-449 bp), 5′-GAAAGTGAGACtaggaGGCTTTCTGCTG-3′ (forward), 5′-CAGCAGAAAGCCtcctaGTCTCACTTTC-3′ (reverse). The lower case letters indicate a deletion mutation. The α7 nAChR gene promoter construct containing the specific site-directed deletion mutation of c-Jun binding site was generated using the QuikChange II Site-Directed Mutagenese Kit according to the manufacturer's recommendations (Stratagene, La Jolla, CA, USA). Briefly, samples containing 50 ng of α7 nAChR promoter (947 bp) dsDNA template and 125 ng of each primer were denatured at 95°C for 30 seconds, followed by 12 cycles (95°C for 30 seconds, 55°C for 1 minute, 68°C for 7 minutes). The amplification products were then digested using *Dpn* I restriction enzyme and transformed into XL1-Blue Supercompetent cells. Colonies were selected and screened for mutants by sequencing using ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

Transient transfection assay

The 947-, 621-, 422-, and 65-bp mouse α7 nAChR promoter deletion constructs (pGL3-α7LUC) ligated to the luciferase reporter gene were a gift from Dr. Stitzel at the University of Colorado and have been reported previously.[18] Briefly, NSCLC cells were seeded at a density of 10^5 cells/well in 24-well plates and grown to 60% confluence. For each well, 0.5 μg of the above α7 nAChR plasmid DNA constructs, with 1 ng of the internal control pRL-CMV Synthetic Renilla Luciferase Reporter Vector (Promega), were cotransfected into the cells using Lipofectamine 2000 reagent (Invitrogen), as described in our earlier study.[19] After 24 hours of incubation, cells were treated with or without $dmPGE_2$ for an additional 24 hours. In separate experiments, cells were transfected with control and c-Jun siRNA (100 nM for each) for 24 hours, before exposing the cells to $dmPGE_2$ for an additional 24 hours. The preparation of cell extracts and the measurement of luciferase activities were carried out using the Dual-Luciferase Reporter Kit according to the manufacturer's recommendations (Promega). The assays for firefly luciferase activity and Renilla luciferase activity were performed sequentially in a Luminoskan Ascent illuminometer (Thermo Labsystems, Helsinki, Finland) equipped with dual injectors. Changes in firefly luciferase activity were calculated

and plotted after normalization with changes in Renilla luciferase activity within the same sample.

Statistical analysis

All experiments were repeated a minimum of three times. All data were expressed as means ± standard deviation. The data presented in some figures was qualitatively representative of replicate experiments. Statistical significance was determined with Student's *t* test (two-tailed) comparison between two groups of data sets. One-way analysis of variance was used for comparison among three or more groups. Asterisks shown in the figures indicate significant differences of experimental groups in comparison with the corresponding control condition ($P < 0.05$, see figure legends).

Results

Prostaglandin E$_2$ (PGE$_2$) increases α7 nAChR gene expression and induces cell growth through α7 nAChR-dependent cholinergic signaling

There are data implicating both PGE$_2$ and cholinergic signaling in the regulation of NSCLC growth. We began by evaluating the effect of PGE$_2$ on α7 nAChR gene expression. We found that PGE$_2$ enhanced the protein level of α7 nAChR in time and dose dependent manners, as determined by Western Blot in H1792 NSCLC cells (Fig. 1a and b). Similar results were observed in several other NSCLC cell lines, but with varying efficiency (Fig. 1c). PGE$_2$ increased the messenger (m)RNA level of α7 nAChR in multiple NSCLC cell lines, determined by real-time RT-PCR (Fig. 1d).

α7 nAChR has been implicated to mediate the effect of nicotine on cell growth.[14] We then tested whether α7 nAChR mediated PGE$_2$-induced cell growth and found that silencing of α7 nAChR significantly diminished the stimulatory effect of PGE$_2$ on the proliferation of H1838 cells, determined by Luminescent Cell Viability assays (Promega) (Fig. 1e). Interestingly, we showed that AChE, which hydrolyzes endogenous ACh (an endogenous ligand for α7 nAChR), also blocked the effect of PGE$_2$ in NSCLC cells (Fig. 1f). In addition to stimulating α7 nAChR expression, these results suggest that the stimulatory effect of PGE$_2$ on cell growth is at least partially mediated by cholinergic signaling. For this reason, we turned our attention to the mechanisms by which PGE$_2$ stimulates α7 nAChR expression.

PGE$_2$ increases α7 nAChR promoter activity

Having established that PGE$_2$ increases α7 nAChR expression, we next examined whether the effects of PGE$_2$ on α7 nAChR expression occur at transcription level. The α7 nAChR gene promoter contains multiple transcription factor binding sites, including c-Jun (Fig. 2a). c-Jun binding sites have been shown to be differentially responsive to various stimuli.[20] We found that H1792 and H1838 cells, transfected with a wild type α7 nAChR gene promoter reporter constructs (Chrna7-947 bp), showed increased promoter activity in response to PGE$_2$ (Fig. 2b). Similar results were found in cells transfected with an α7 nAChR gene reporter promoter deletion construct (Chrna7-621 bp) (Fig. 2c). However, this effect was not observed when testing two smaller α7 nAChR promoter reporter constructs (Chrna7-422 bp and −65bp), in which a specific c-Jun binding site (-455/-449 bp) in the upstream region of α7 nAChR gene was missing (Fig. 2c). Furthermore, we showed that PGE$_2$-induced α7 nAChR promoter activity was eliminated in cells transfected with an α7 nAChR promoter reporter construct in which this c-Jun site (-455/-449 bp) was mutated (Fig. 2d).

PGE$_2$ increases α7 nAChR gene expression via EP4, PI3-K, PKA, and JNK signals

To test whether PGE$_2$ affects α7 nAChR through its EP4 receptor, we used a selective EP4 antagonist, AH23848, to block EP4 function. We showed that AH23848 abrogated the effect of PGE$_2$ on induction of α7 nAChR protein expression in H1792 cells (Fig. 3a). This was further confirmed by using cells transfected with EP4 siRNA (Fig. 3b). Silencing EP4 expression blocked the effect of PGE$_2$ on induction of α7 nAChR protein expression, while the control siRNA had no effect (Fig. 3b). Cell viability assays showed that AH23848 blocked the stimulatory effect of PGE$_2$ on cell proliferation in H1838 and H1792 cells (Fig. 3c).

Multiple kinase signals are reported to mediate the effect of PGE$_2$ on carcinoma cell growth.[21,22] Here we show that PGE$_2$ induced the phosphorylation of JNK in a time-dependent manner with maximal stimulation in half an hour at 1 μM in H1792 and H1838 cells (Fig. 3d). Total JNK protein was not affected (Fig. 3d).

We therefore tested whether kinase pathways mediated the regulation of α7 nAChR expression. Western blot analysis revealed that PGE$_2$-induced α7 nAChR protein expression was reduced in the presence of the PKA inhibitor, H-89 (10 μM) (Fig. 3e), and JNK inhibitor II (20 μM) (Fig. 3f). The PI3-K inhibitors, Wortmannin (1 μM) and LY294002 (10 μM) also partially inhibited PGE$_2$-induced α7 nAChR protein expression (Fig. 3g). The findings above indicate that the stimulatory effect of PGE$_2$ on α7 nAChR is associated with the activation of the PKA, JNK, and PI3-K signaling pathways. In contrast, the inhibitor of ERK, PD98059 (10 μM) had no effect (Fig. 3e).

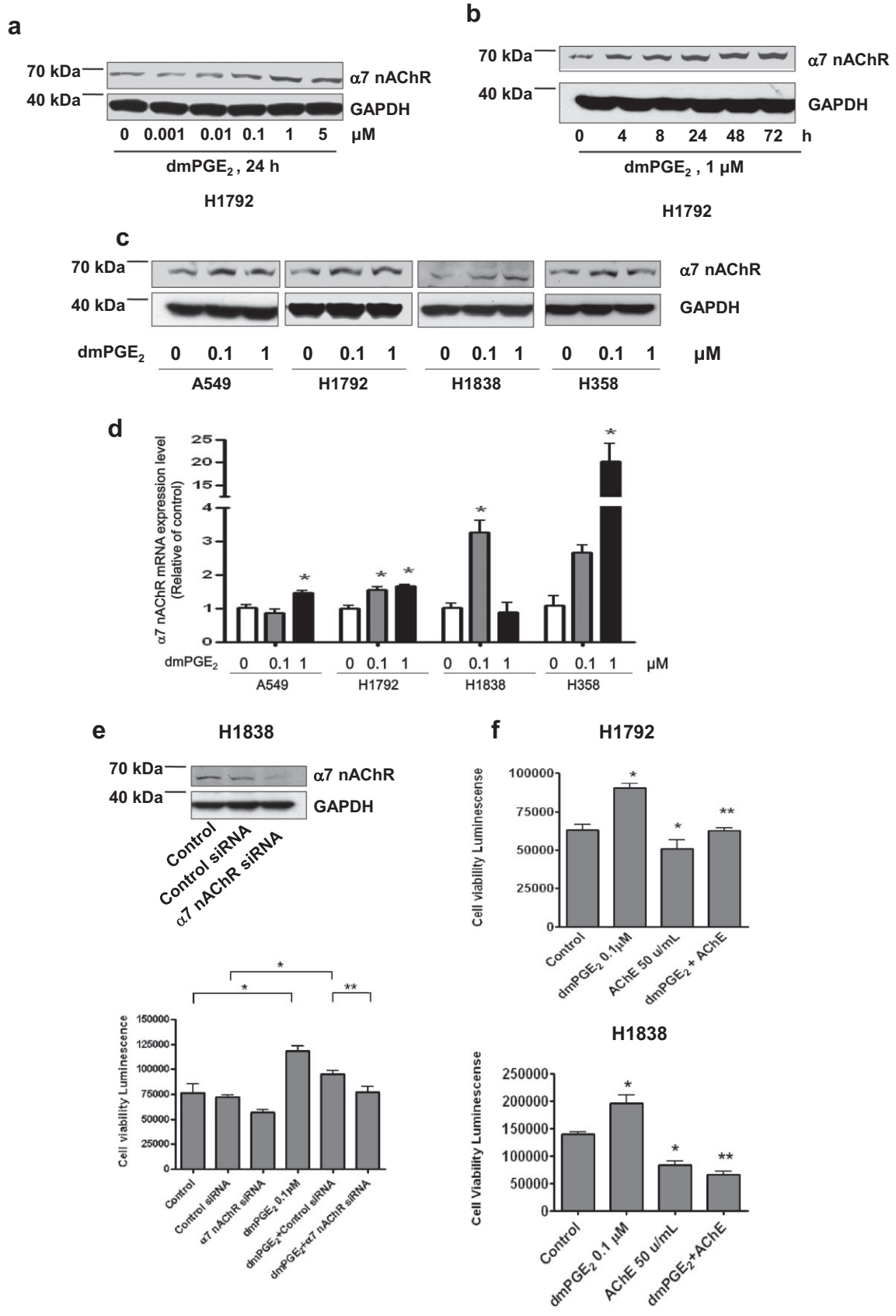

Figure 1 Prostaglandin E_2 (PGE_2) increases α7 nicotinic acetylcholine receptor (nAChR) protein expression and induces cell growth partly through α7 nAChR-dependent cholinergic signaling. (a) Cellular protein was isolated from H1792 cells that were cultured with increased concentrations of $dmPGE_2$ as indicated for 24 hours followed by Western blot analysis with antibodies against α7 nAChR protein. (b) Cellular protein was isolated from H1792 cells that were cultured with $dmPGE_2$ (1 μM) for the indicated time, followed by Western blot analysis with antibodies against α7 nAChR protein. (c) Cellular protein was isolated from several non-small cell lung cancer (NSCLC) cell lines (A549, H1792, H1838, and H358) that were cultured with $dmPGE_2$ (0.1, 1 μM) for up to 24 hours, followed by Western blot analysis with antibodies against α7 nAChR protein. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an internal control for normalization purposes. (d) Total ribonucleic acid (RNA) was isolated from several NSCLC cell lines (A549, H1792, H1838, and H358) treated with $dmPGE_2$ (0.1, 1 μM) for up to 24 hours and real time reverse transcription polymerase chain reaction was performed for evaluating α7 nAChR messenger RNA expression. GAPDH served as an internal control for normalization purposes. (e) H1838 cells were transfected with control or α7 nAChR small interfering RNA (100 nM) for 24 hours before exposure of the cells to $dmPGE_2$ (0.1 μM) for up to three days. The viable cells were then determined by Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega). (f) H1792 and H1838 cells were cultured with $dmPGE_2$ (0.1 μM) in the presence or absence of AChE (50 U/mL) for up to five days. The viable cells were then detected using the Cell Titer-Glo Luminescent Cell Viability Assay kit. All data were depicted as mean ± standard deviation. * indicates significant difference compared to the untreated cell group. ** indicates significant difference of combination treatment compared to $dmPGE_2$ alone ($P < 0.05$).

PGE_2 induces c-Jun protein expression via PI3-K, PKA, and JNK signals

Results obtained in cells transfected with the deletion and mutation constructs of the α7 nAChR gene promoter strongly suggested the importance of the presence of a c-Jun site in the proximal portion of the α7 nAChR promoter.[23] Therefore, we further tested the role of c-Jun in this process. We showed that PGE_2 induced protein expression of c-Jun in a dose-dependent manner (Fig. 4a). Consistent with our prior data, this effect of PGE_2 on c-Jun expression was abrogated by H-89 (10 μM) and JNK inhibitor II (20 μM), partially blocked by Wortmannin (1 μM) and LY294002 (10 μM), but not by PD98059 (10 μM) (Fig. 4b–d). The findings suggested that PI3-K/AKT, PKA, and JNK signaling pathways are involved in the induction of c-Jun by PGE_2.

The role of c-Jun in mediating the effect of PGE_2 on α7 nAChR expression

The above results implicate c-Jun in the stimulatory effect of PGE_2 on α7 nAChR expression. We further tested this by silencing the c-Jun gene by siRNA. As expected, silencing the c-Jun blocked the stimulatory effect of PGE_2 on α7 nAChR protein expression, while there was no effect on the control siRNA (Fig. 5a). Furthermore, c-Jun siRNA also greatly reduced the stimulatory effect of PGE_2 on α7 nAChR promoter activity, while there was a small effect on the control siRNA (Fig. 5b). In addition, knockdown of the c-Jun gene antagonized the stimulatory effect of PGE_2 on cell growth, determined by cell viability assays (Fig. 5c). Note that there was no effect on the control siRNA, while the c-Jun siRNA abrogated the expression of c-Jun protein (Fig. 5c, upper panel).

Discussion

Both prostanoid signaling and α7 nAChR-dependent cholinergic signaling have significant roles in cancer growth, apoptosis, progression, and metastasis.[8,24,25] Recently, increasing efforts have been made to identify their underlying molecular mechanisms with the objective of identifying safer strategies for cancer treatment.[2] PGE_2 has been shown to enhance tumor growth and apoptosis resistance, and increase tumor vascularization in several systems, including lung cancer.[9,11,26] Activated non-neuronal cholinergic signaling has also been implicated in human lung cancer survival.[12,13] Among the nAChRs, α7 nAChR has received the most attention. Studies from our laboratory and others have demonstrated that nicotine, the major pharmacologically active substance in cigarette smoke, stimulates lung cancer cell growth through α7 nAChR-mediated signals.[14] Moreover, inhibition of non-neuronal α7 nAChR reduces tumorigenicity in NSCLC xenografts.[24] The α7 nAChR antagonist has been shown to inhibit angiogenesis in lung cancer.[27]

Several pieces of evidence have suggested a link between prostanoid and cholinergic signaling in the process of cancer growth.[28,29] Nicotine has been reported to induce COX-2 expression and PGE_2 production in several tumor cells, partly through α7 nAChR and other signaling pathways, and a blockade of COX-2/PGE_2 signaling prevented nicotine-induced tumor growth and neovascularization.[28–30] The nicotine derivative, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), induces COX-2/PGE_2 production and gastric cancer cell proliferation, which is blocked by an α7 nAChR inhibitor.[28] Therefore, we explored the link between PGE_2 and α7 nAChR-dependent cholinergic signaling in lung cancer cell proliferation. We found that PGE_2 induces α7 nAChR gene expression. The stimulatory effect of PGE_2 on cell proliferation was significantly diminished when we knocked down the expression of the receptor α7 nAChR or hydrolyzed the endogenous α7 nAChR ligand ACh with AChE. Our results demonstrate a cross talk between PGE_2 and α7 nAChR, and imply that PGE_2 may promote proliferation through its effects on α7 nAChR. Thus, α7 nAChR might represent a common novel target for blocking both prostanoid and cholinergic signaling pathways in lung cancer.

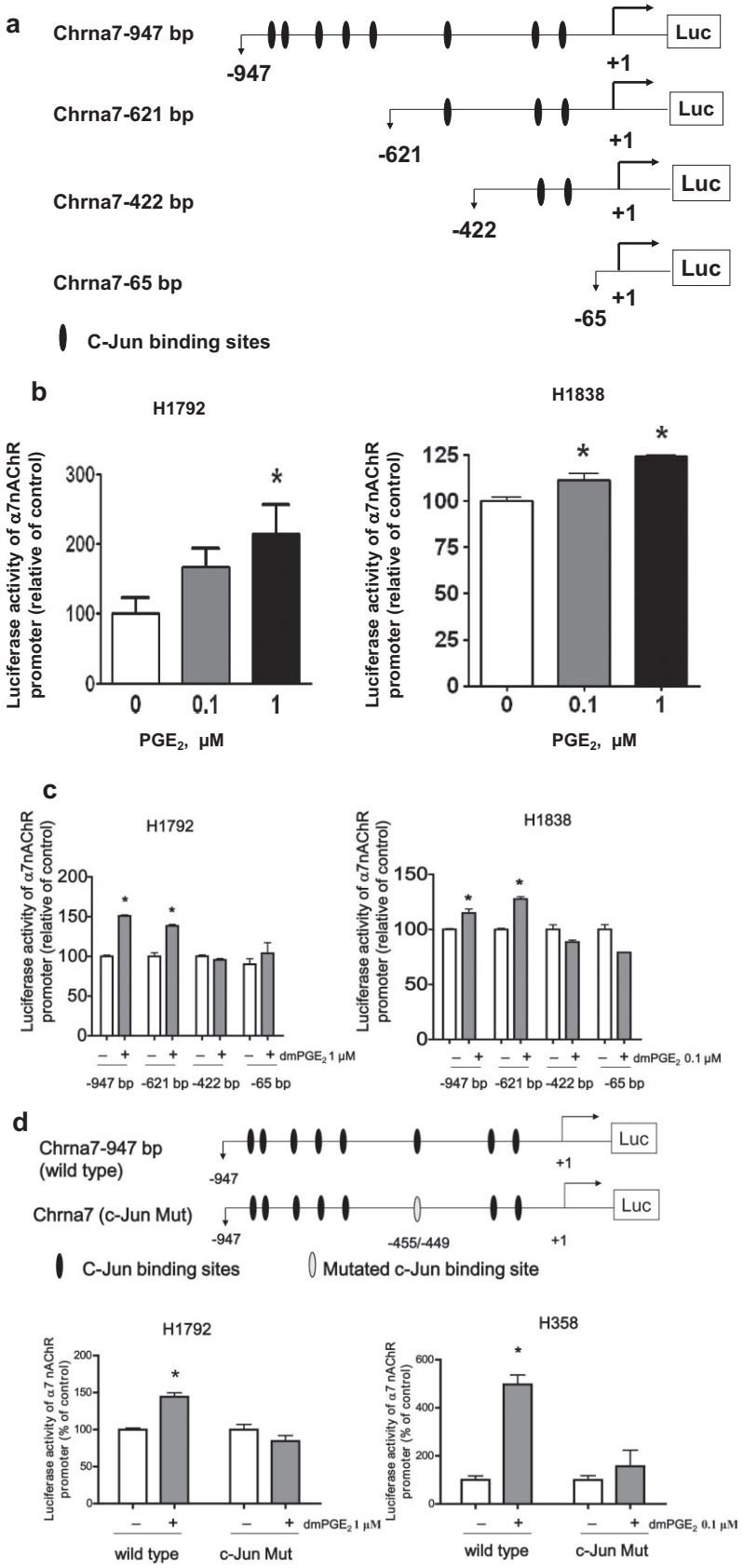

Figure 2 Prostaglandin E_2 (PGE_2) increases α7 nicotinic acetylcholine receptor (nAChR) promoter activity. (a) The mouse Chrna7 wild type and deletion promoter construct schematics are presented. These regions contain several transcription factor-binding sites, including c-Jun/AP-1. (b) H1792 and H1838 cells ($1x10^5$ cells) were co-transfected with a wild type mouse full-length Chrna7 promoter reporter construct (shown in a) ligated to a luciferase reporter gene and an internal control Renilla Luciferase Reporter Vector for 24 hours using the oligofectamine reagent according to the manufacturer's instructions. After 24 hours of incubation, H1792 and H1838 cells were treated with 1 μM and 0.1 μM $dmPGE_2$ respectively for an additional 24 hours. A Dual Luciferase Reporter kit (Promega) determined luciferase activity. (c) H1792 and H1838 cells ($1x10^5$ cells) were co-transfected with a wild type full-length and several deletion mouse Chrna7 promoter reporter constructs (shown in a) ligated to a luciferase reporter gene and an internal control Renilla Luciferase Reporter Vector (Promega) for 24 hours. After 24 hours of incubation, H1792 and H1838 cells were treated with 1 μM and 0.1 μM $dmPGE_2$ respectively for an additional 24 hours. A Dual Luciferase Reporter kit determined luciferase activity. *Control,* indicates untreated control cells. (d) H1792 and H358 cells were transfected with a wild type Chrna7 (Chrna7-947 bp) or c-Jun mutation promoter reporter construct (shown in d, upper panel) and an internal control for 24 hours, then cells were exposed to $dmPGE_2$ (0.1, 1 μM) for an additional 24 hours. Luciferase activity was determined by a Dual Luciferase Reporter kit. * indicates significant difference compared to the untreated cell group ($P < 0.05$).

The concentrations of PGE_2 used in this study were based on our previous work showing that fibronectin, a matrix glycoprotein, stimulates lung cancer growth and diminishes apoptosis via upregulation of COX-2 expression and PGE_2 biosynthesis.[19] Similar or even smaller doses of exogenous PGE_2 have been implicated to have a mitogenic effect on colorectal carcinoma cells.[31] The cellular effects of PGE_2 are mediated through a family of G-protein–coupled receptors designated EP 1, 2, 3, and 4.[22] Among them, EP4 has been implicated in tumor cell proliferation, cell cycle control, migration, and progression.[21] One recent study showed that EP4 mediates PGE_2-induced A549 lung cancer cell migration.[9] In this study, we demonstrated the critical role of EP4 in mediating the effect of PGE_2 on α7 nAChR protein expression and cell growth.

The intracellular pathways mediating the effect of PGE_2 on α7 nAChR expression in NSCLC have not been elucidated. The PI3-K/Akt, PKA, JNK, and ERK pathways are critical pathways in cancer because they contribute to tumor growth, survival, invasion, metastasis, tumor angiogenesis, and resistance to therapy.[26,32,33] PGE_2 has been shown to stimulate tumor cell growth through activation of PI3-K/Akt, PKA, JNK, and ERK signal pathways in several studies.[34–36] Here we have shown that PGE_2 acting on EP4 receptors induced the phosphorylation of JNK in NSCLC cells. JNK has been found to play a pivotal role in activating transcription factors (including c-Jun) that increase cellular growth and tumor formation.[32] Our results indicate that activation of PI3-K, PKA, and JNK is involved in the effect of PGE_2 on α7 nAChR expression, while ERK was less effective.

We then investigated if PGE_2 induced-α7 nAChR expression reflected enhanced transactivation of the α7 nAChR gene. We showed that activated AP-1 subunit c-Jun binding to the downstream areas of the α7 nAChR gene promoter played a role in mediating the stimulatory effect of PGE_2 on α7 nAChR expression. Furthermore, our results confirmed an important role of c-Jun in mediating the effect of PGE_2 on α7 nAChR expression and cell growth. Data regarding AP-1/c-Jun in regulation of α7 nAChR expression are scarce. AP-1 activity is increased in multiple human tumor types, including lung cancer.[37] Inhibitors of AP-1 have been shown to block tumor promotion, transformation, progression, and invasion.[37] In addition, AP-1 is a recognized molecular target of many antioxidant and anti-inflammatory chemopreventive compounds. PGE_2 has recently been shown to activate AP-1 through its EP receptors in human prostate cancer cells.[38] c-Jun, a major constituent of AP-1, is overexpressed in NSCLC cells.[39] The blockade of c-Jun by overexpression of a c-Jun dominant-negative mutant, TAM67, inhibited NSCLC cell growth.[39] Similarly, overexpression of c-Jun in MCF-7 cells resulted in increased AP-1 activity, enhanced motility and invasiveness of the cells *in vitro,* and increased tumor formation in nude mice.[40] PGE_2 has been reported to stimulate c-Jun in breast adipose fibroblasts.[36] In addition, exogenous PGE_2, as well as COX-2 overexpression, affects c-Jun expression, and enhances colon cancer cell progression.[41] Our data showing the effects of silencing c-Jun, together with point mutation analysis, point to a key role for c-Jun transactivation in the regulation of α7 nAChR gene promoter activity and PGE_2-related cell growth.

Conclusion

Our data suggest that PGE_2 increases α7 nAChR expression through EP4-mediated induction of c-Jun protein expression and increased activity of the α7 nAChR gene promoter. By upregulating the nicotinic receptor for endogenous non-neuronal ACh, PGE_2 activates cholinergic signaling and promotes NSCLC cell growth (Fig. 6). To our knowledge, this is the first demonstration of a link between PGE_2 and α7 nAChR signaling in human lung carcinoma cells. We identified critical cell signaling pathways (JNK, PI3-K, and PKA) and a key transcription factor (c-Jun). These findings open up new potential molecular targets for intervention in NSCLC.

Acknowledgments

We are grateful to Dr. Jerry A. Stitzel at University of Colorado for providing the α7 nAChR promoter constructs. This work was supported by the National Institute of Health (Grant

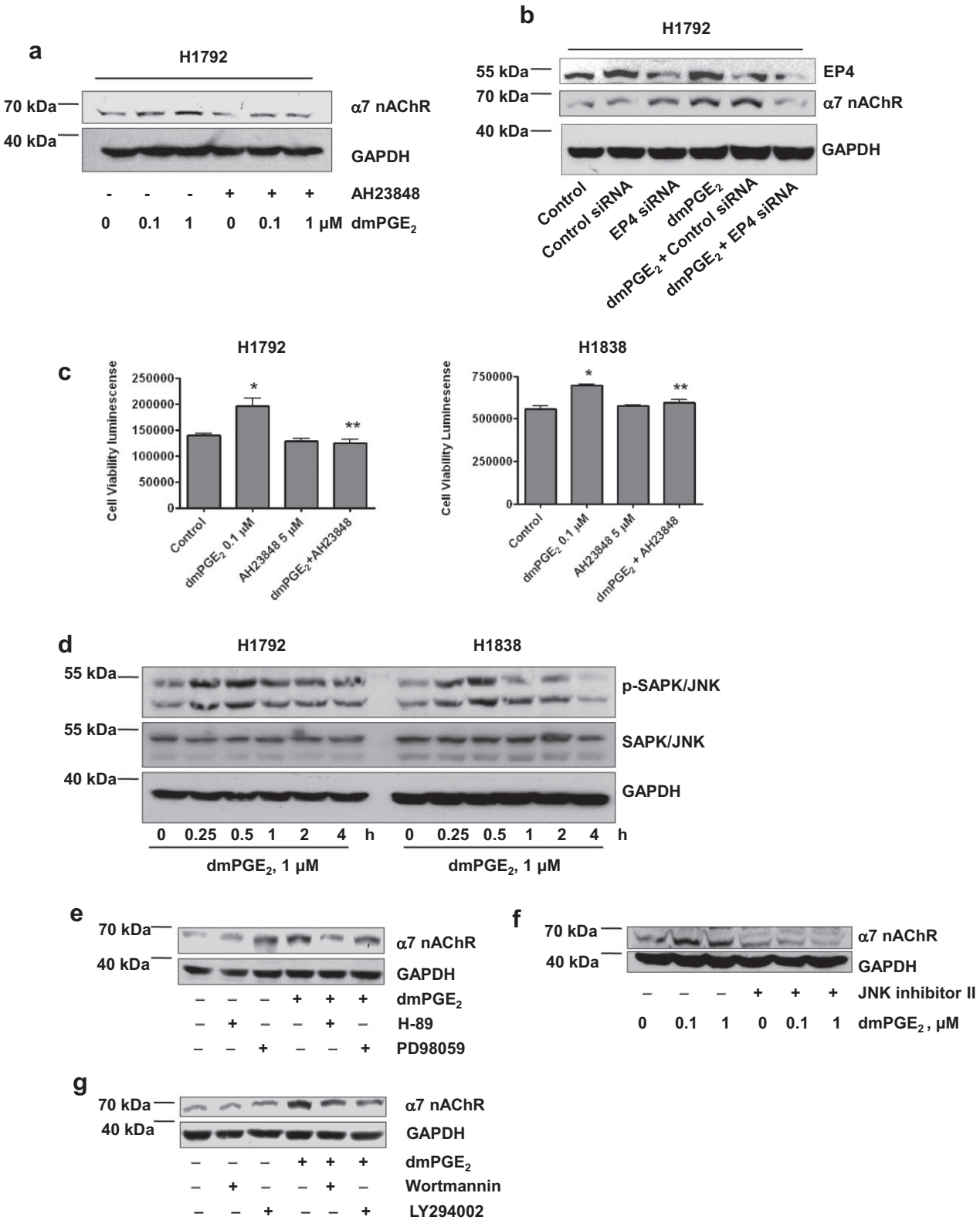

Figure 3 Prostaglandin E$_2$ (PGE$_2$) increases α7 nicotinic acetylcholine receptor (nAChR) gene expression via EP4 and phosphatidylinositol 3-kinase (PI3-K), protein kinase A (PKA), and c-Jun N-terminal kinase (JNK) signals. (a) Cellular protein was isolated from H1792 cells cultured for up to two hours in the presence or absence of AH23848 (5 μM) before exposure of cells to dmPGE$_2$ (0.1, 1 μM) for an additional 48 hours, then subjected to Western blot analysis for α7 nAChR protein. (b) Cellular protein was isolated from H1792 cells cultured for 24 hours in the presence or absence of the control or EP4 small interfering ribonucleic acid (100 nM) before exposure of cells to dmPGE$_2$ (1 μM) for an additional 24 hours, and then subjected to Western blot analysis for EP4 and α7 nAChR protein. (c) H1792 and H1838 cells were cultured with dmPGE$_2$ (0.1 μM) in the presence or absence of AH23848 (5 μM) for up to five days. The viable cells were then detected using a Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega). (d) Cellular protein was isolated from H1838 and H1792 cells treated with dmPGE$_2$ (1 μM) for the indicated time followed by Western blot analysis with antibodies against phospho-SAPK/JNK and SAPK/JNK proteins. (e) Cellular protein was isolated from H1792 cells treated with H-89 (10 μM) or PD98059 (10 μM) for two hours before exposure of the cells to dmPGE$_2$ (1 μM) for an additional 48 hours, then subjected to Western Blot analysis for α7 nAChR protein. (f) Cellular protein was isolated from H1792 cells treated with JNK inhibitor II (20 μM) for two hours before exposure of the cells to dmPGE$_2$ (0.1, 1 μM) for an additional 48 hours, then subjected to Western Blot analysis for α7 nAChR protein. (g) Cellular protein was isolated from H1792 cells treated with Wortmannin (1 μM) or LY294002 (10 μM) for two hours before exposure of the cells to dmPGE$_2$ (1 μM) for an additional 48 hours, then subjected to Western Blot analysis for α7 nAChR protein. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as internal control for loading purposes. *Control*, indicates untreated control cells. * indicates significant difference compared to the untreated cell group. ** indicates significant difference of combination treatment as compared to the dmPGE$_2$ alone ($P < 0.05$).

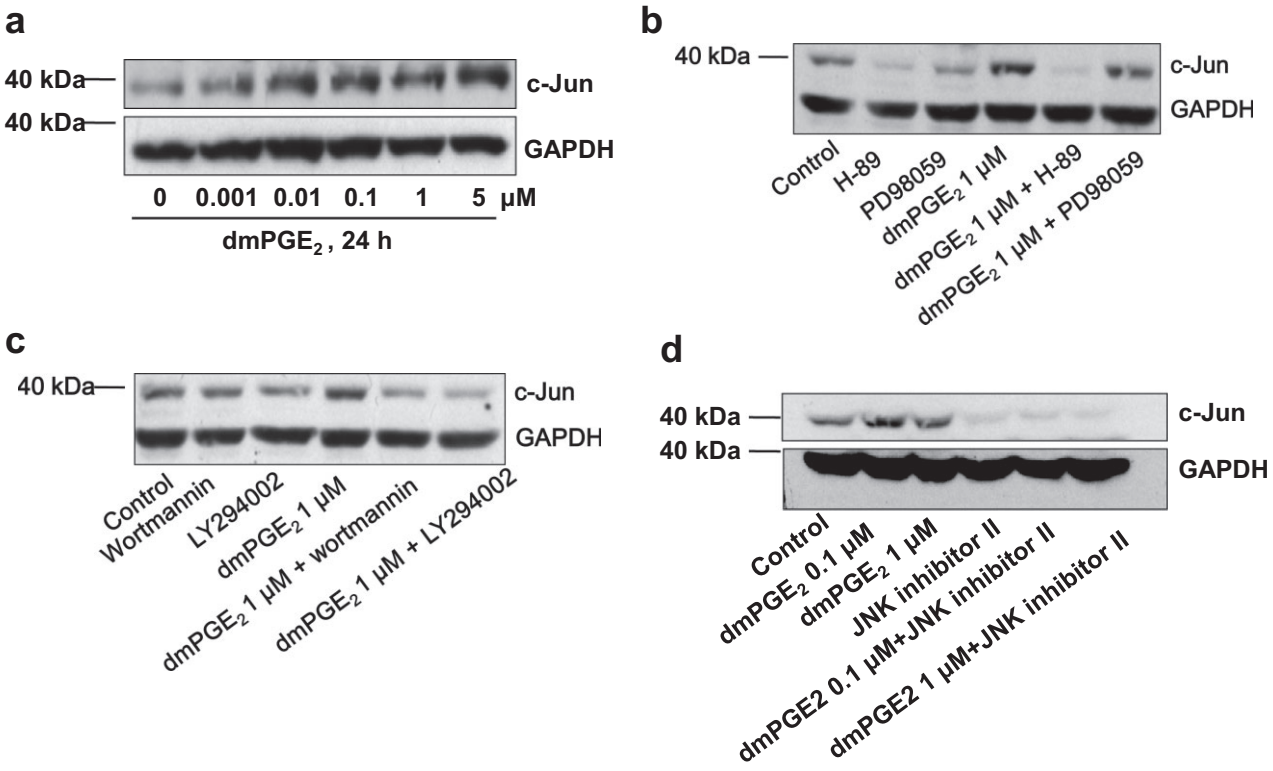

Figure 4 Prostaglandin E$_2$ (PGE$_2$) induces c-Jun protein expression via phosphatidylinositol 3-kinase (PI3-K), protein kinase A (PKA), and c-Jun N-terminal kinase (JNK) signals. (a) Cellular proteins were isolated from H1792 cells treated with increased concentrations of dmPGE$_2$ for 24 hours. Afterwards, Western Blot analyses were performed using a polyclonal antibody against c-Jun protein. (b–d) Cellular protein was isolated from H1792 cells treated with H-89 (10 μM), PD98059 (10 μM), Wortmannin (1 μM), LY294002 (10 μM), or JNK inhibitor II (20 μM) for two hours before exposure of the cells to dmPGE$_2$ (0.1, 1 μM) for an additional 48 hours, then subjected to Western blot analysis for c-Jun protein. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control for loading purposes. *Control*, indicates untreated control cells.

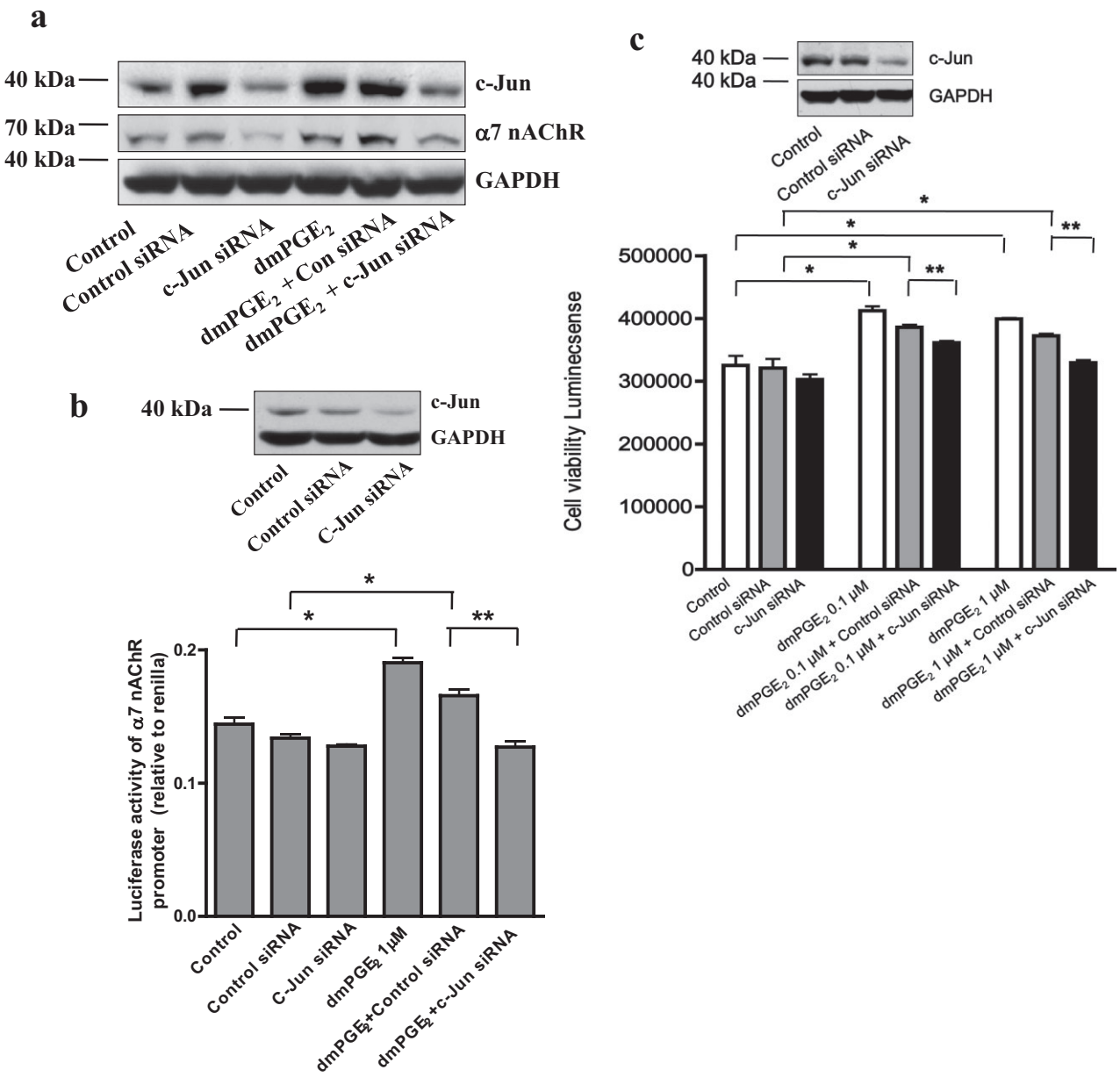

Figure 5 The role of c-Jun in mediating the effect of prostaglandin E_2 (PGE_2) on α7 nicotinic acetylcholine receptor (nAChR) expression and cell growth. (a) H1792 cells were transfected with control or c-Jun small interfering ribonucleic acid (siRNA) (100 nM) for 24 hours before exposure of the cells to $dmPGE_2$ (1 μM) for an additional 48 hours. Western blot analysis was then performed to examine for α7 nAChR and c-Jun protein. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as loading control. (b) H1792 cells were transfected with control or c-Jun siRNAs (100 nM) together with a wild type Chrna7 promoter reporter construct ligated to a luciferase reporter gene and an internal control for 24 hours. Cells were then exposed to $dmPGE_2$ (1 μM) for an additional 24 hours. A Dual Luciferase Reporter kit (Promega) determined luciferase activity. The insert in the upper panel represents Western blot results for c-Jun protein. GAPDH served as internal control for normalization purposes. (c) H1838 cells were transfected with control or c-Jun siRNAs (100 nM) for 24 hours. Cells were then exposed to $dmPGE_2$ (0.1, 1 μM) for an additional 72 hours. A Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega) determined cell numbers. The insert in the upper panel represents Western blot results for c-Jun protein. GAPDH served as internal control for normalization purposes. The bars below represent the mean ± standard deviation of at least three independent experiments for each condition. * indicates significant increase of activity compared to controls. ** indicates significance of combination treatment compared to $dmPGE_2$ alone ($P < 0.05$). *Control*, indicates untreated control cells.

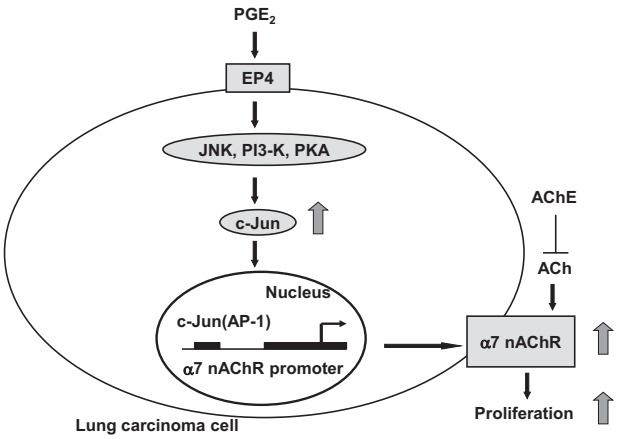

Figure 6 The schematic representation of the role of c-Jun in prostaglandin E_2 (PGE_2)-induced α7 nicotinic acetylcholine receptor (nAChR expression and tumor cell proliferation. The diagram shows that PGE_2 induces α7 nAChR via activation of c-Jun N-terminal kinase (JNK), phosphatidylinositol 3-kinase (PI3-K) and protein kinase A (PKA), followed by increasing c-Jun expression. PGE_2-induced α7 nAChR then activates ACh/α7 nAChR signaling and leads to human lung cancer cell growth.

#CA116812); and an Intramural Research Incentive Grant from the Office of the Executive Vice President for Research at the University of Louisville.

Disclosure

No authors report any conflict of interest.

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