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15-Keto Prostaglandin E₂ Inhibits
Proliferation of Breast Cancer Cells through
Suppression of STAT3 Signaling

STAT3 억제를 통한 15-Keto prostaglandin E₂의
인체 유방암세포 증식 억제 연구

2017 년 2 월

서울대학교 대학원
분자의학 및 바이오제약학과
이 은 지
Abstract

15-Keto Prostaglandin E₂ Inhibits Proliferation of Breast Cancer Cells through Suppression of STAT3 Signaling

Eun Ji Lee
Under the supervision of Professor Young-Joon Surh
Department of Molecular Medicine and Biopharmaceutical Science
Seoul National University

Prostaglandin E₂ (PGE₂) is highly produced during inflammation and cancer. Overproduction of PGE₂ has been associated with increased tumor cell proliferation, resistance to apoptosis and invasiveness. PGE₂ is oxidized to 15-keto prostaglandin E₂ (15-keto PGE₂) by 15-hydroxyprostaglandin dehydrogenase (15-PGDH). 15-Keto PGE₂ is considered as a biologically inactive form of the pro-tumorigenic lipid mediator, PGE₂. However, recent studies have revealed that 15-keto PGE₂ has tumor suppressive functions, but the underlying molecular mechanisms remain to be elucidated. Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that regulates transcription of genes involved in cell proliferation, cell cycle progression and cell survival. It is constitutively activated in most of cancer types, and the aberrantly activated STAT3 contributes to tumor promotion and progression. This prompted me to investigate the effects of 15-keto PGE₂ on STAT3 activation. Notably, 15-keto PGE₂ containing an α,β-unsaturated carbonyl group directly bound to STAT3 and thereby...
suppressed the phosphorylation, dimerization and nuclear translocation of this transcription factor in Ha-Ras transformed human mammary epithelial (MCF10A-ras) cells. In contrast, its non-electrophilic analogue, 13,14-dihydro-15-keto PGE2 failed to inhibit the STAT3 activation and was unable to suppress the growth and transformation of MCF10A-ras cells. Moreover, thiol reducing agents, such as dithiothreitol or N-acetyl-L-cysteine, disrupted the interaction between 15-keto PGE2 and STAT3, and abrogated STAT3 inactivation by 15-keto PGE2. A molecular docking analysis suggests that Cys\textsuperscript{251} and Cys\textsuperscript{259} residues of STAT3 are preferential binding sites for this lipid mediator. In addition, subcutaneous injection of 15-keto PGE2 attenuated xenograft tumor growth and decreased phosphorylated STAT3 in athymic nude mice transplanted with human breast cancer MDA-MB-231 cells. Taken together, thiol modification of STAT3 by 15-keto PGE2 inhibits STAT3 activation, thereby suppressing breast cancer cell proliferation, growth and transformation.

**Keywords:** 15-Keto PGE\textsubscript{2}, electrophile, $\alpha$,\$^{-}$-unsaturated carbonyl group, STAT3, thiol modification, MCF10A-ras, MDA-MB-231 xenografts

**Student Number:** 2015-26014
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**Introduction**

Prostaglandin E$_2$ (PGE$_2$) is an inflammatory lipid mediator produced as one of the major products of the arachidonic acid metabolism catalyzed by cyclooxygenase-2 (COX-2) during inflammation [1]. Production of PGE$_2$ increases upon inflammatory insults, and its levels are abnormally elevated in various human malignancies [2, 3] including breast cancer [4]. There is a close association between inflammation and cancer [5]. COX-2-derived PGE$_2$ has been known to play a key role in inflammation-associated cancer progression [6].

PGE$_2$ exerts its function by binding to specific cell surface receptors, called EP receptors [7, 8]. PGE$_2$-induced overactivation of EP receptors can accelerate cancer progression through modulation of several intracellular signaling pathways [9-11]. Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that regulates cellular processes involved in proliferation, development, inflammation and cell survival [12]. In response to extracellular stimuli, such as cytokines and growth factors, STAT3 is recruited from the cytosol to their receptors and is phosphorylated by receptor-associated janus kinase (JAKs) [13]. Phosphorylation of the tyrosine 705 residue of STAT3 induces a dimer formation and, then the resulting dimer complex translocates to the nucleus for initiating transcription of target genes [14]. Aberrant activation of STAT3 is linked to tumorigenesis [15]. In contrast to the transient STAT3 activation in normal cells, most of cancer cells retain constitutively activated STAT3 activity [16]. For instance, STAT3 is overactivated in more than 40% of breast cancers [17]. Therefore, targeting abnormally overactivated STAT3 signal transduction has been suggested as a therapeutic strategy in the management of cancer [16, 18, 19]. Many
studies revealed a positive correlation between PGE$_2$ overproduction and STAT3 overactivation in prostate, lung, bladder and colorectal cancers [20-22]. Therefore, it is likely that the PGE$_2$-STAT3 axis has a crucial role in tumorigenesis.

PGE$_2$ undergoes oxidation by 15-hydroxyprostaglandin dehydrogenase (15-PGDH) to produce 15-keto PGE$_2$ which has been considered biologically inactive [23]. However, recent studies have revealed that 15-keto PGE$_2$ has antitumor functions. Thus, 15-keto PGE$_2$ has been reported to induce apoptosis of pancreatic adenocarcinoma through production of ROS [24]. The 15-keto PGE$_2$-induced oxidative stress was attributable to its suppression of expression of $\lambda$CT and CTH encoding, respectively a light-chain subunit of cysteine-glutamate exchange transporter and cystathionine $\gamma$-lyase that play key roles in providing cellular cysteine supply for glutathione biosynthesis. The cell death-promoting effect of 15-keto-PGE$_2$ is mediated partly but not entirely through PPAR$\gamma$ activation [24]. In another study, 15-keto PGE$_2$ was demonstrated to inhibit hepatocellular carcinoma growth through PPAR$\gamma$-mediated activation of p21$^{\text{WAF/Cip1}}$ [25]. Thus, the mechanisms underlying cytotoxic effects of 15-keto PGE$_2$ appear to be different, depending on the cancer cell type.

15-Keto PGE$_2$ has an $\alpha,\beta$-unsaturated carbonyl moiety [26], and hence can act as a Michael addition acceptor. The electrophilic $\alpha,\beta$-unsaturated carbonyl group modifies the nucleophilic thiol group of cysteine residue(s) in various proteins [27-29]. For instance, 15-keto PGE$_2$ can act as a PPAR$\gamma$ ligand through the Michael addition to the cysteine residue of this transcription factor [26, 30]. Similarly, cysteine residues contained in STAT3 are speculated targets of 15-keto PGE$_2$. In this study, I investigated whether 15-keto PGE$_2$ could bind and subsequently inactivate STAT3, thereby suppressing human breast cancer cell proliferation and growth. I found that the
electrophilic α,β-unsaturated carbonyl group of 15-keto PGE₂ covalently modifies STAT3 thiol residue(s) and prevents STAT3 activation.
Materials and Methods

Cell culture

MCF10A and MCF10A-ras cells were cultured in Dulbecco’s Modified Eagle Medium: Nutrient Mixture-F-12 (DMEM/F-12) supplemented with 5% heat-inactivated horse serum from Gibco (Grand Island, NY, USA), 10 μg/ml insulin, 100 ng/ml cholera toxin, 0.5 μg/ml hydrocortisone, 20 ng/ml human epidermal growth factor, 2 mmol/L L-glutamine and 100 units/ml penicillin/streptomycin. MDA-MB-231 and HeLa/P-STAT3-Luc cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) from GenDEPOT (Barker, TX, USA) and 1% antibiotic-antimycotic mixture. PC3 cells were cultivated in RPMI 1640 containing 10% heat-inactivated FBS from GenDEPOT and 1% antibiotic-antimycotic mixture. These cell lines were grown at 37°C in humidified atmosphere of 5% CO₂.

Chemicals

15-Keto PGE₂ (9,15-dioxo-11α-hydroxy-prosta-5Z,13E-dien-1-oic acid) and 13-14-dihydro-15-keto PGE₂ (9,15-dioxo-11α-hydroxy-prosta-5Z-en-1-oic acid) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Dithiothreitol (DTT) and N-acetyl-L-cysteine (NAC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Primary antibodies for STAT3, P-STAT3 Y705, JAK2 and P-JAK2 were supplied from Cell Signaling Technology (Danvers, MA, USA). Actin and lamin B antibodies were supplied from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 15-PGDH primary antibody was obtained from Cayman Chemicals. Anti-rabbit and anti-mouse horseradish peroxidase conjugated secondary antibodies were purchased from Zymed.
Western Blot Analysis

Preparation of cell lysates

After treatment with 15-keto PGE$_2$ or 13,14-dihydro-15-keto PGE$_2$, MCF10A-ras cells were harvested at indicated time points. The cells were rinsed with cold phosphate-buffered saline (PBS) and then scraped in 1 ml PBS followed by centrifugation at 1,700 g for 5 min at 4°C. Whole cell lysates were prepared with 10x Cell Lysis Buffer purchased from Cell Signaling Technology diluted to 1x solution containing 1% of phenylmethylsulfonyl fluoride (PMSF). Cell pellets were resuspended and incubated for 1 h on ice followed by centrifugation at 18,000 g for 15 min. Supernatant was collected as whole cell lysates. For obtaining cytosolic and nuclear extracts, buffer A was used for cytosolic extracts and buffer C for nuclear extracts. Pellets were resuspended in hypotonic buffer A [10mM HEPES (pH 7.9), 1.5 mM MgCl$_2$, 10 mM KCl, 0.5 mM DTT and 0.2 mM PMSF] for 15 min on ice, and 0.1% Nonidet P-40 (NP-40) was added for less than 5 min. The mixture was then centrifuged at 6,000 g for 5 min at 4°C. The supernatant contained cytosolic protein. The pellets were rinsed twice with hypotonic buffer A and resuspended again in hypertonic buffer C [20 mM HEPES (pH 7.9), 1.5 mM MgCl$_2$, 420 mM NaCl, 0.5 mM DTT, 0.2 mM PMSF, 0.2 mM EDTA and 20% glycerol]. After incubation for 1 h on ice, the mixtures were centrifuged at 18,000 g for 15 min at 4°C. Supernatant was collected as nuclear extract.

Quantification of the protein concentration

Protein concentrations in whole cell lysates were determined by using the BCA
protein assay kit supplied from ThermoFisher Scientific (Rockford, IL, USA). Protein concentrations in cytosolic and nuclear extracts were determined by using the Bradford Assay Reagents kit purchased from Bio-Rad (Hercules, CA, USA).

**Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

Equivalent amounts of protein from whole cell lysate or nuclear fraction were mixed with SDS sample loading dye and boiled for 5 min at 99°C. Protein samples were resolved by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) microporous membrane supplied from PALL Corporation (Port Washington, NY, USA). Non-specific antibody binding sites were blocked by 3% skim milk in PBS containing 0.05% Tween 20 (PBST) for 1 h at room temperature. Membranes were washed and followed by incubation in PBST containing specific primary antibodies for each condition. The membranes were rinsed three times again and incubated with respective horseradish peroxidase conjugated secondary antibodies for 1 h at room temperature. Peroxidase activity was detected by incubating in ECL reagent for 5 min according to the manufacturer’s instruction and visualized using the imagequant LAS-4000 purchased from Fujifilm Life Science (Stamford, CT, USA)

**Reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was isolated from MCF10A-ras cells by using TRIzol® supplied from Invitrogen (Carlsbad, CA, USA) according to the manufacturer’s protocol. To generate cDNA, 1 μg of total RNA was reverse transcribed with murine leukemia virus reverse transcriptase purchased from Promega (Madison, WI, USA) for 50 min at 42°C and again for 15 min at 72°C. The cDNA sample (1 μl) was amplified in sequential reactions.
The mRNA expression of GAPDH and STAT3 target genes (MCP-1, CCL-5, Cyclin D1, p21 and Bcl-xL) was determined according to following conditions.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sequence</th>
<th>Cycles</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward 5'-GCA TGG CCT TCC GTG TCC CC-3’</td>
<td>28</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-CAA TGC TGG CCC CAG CTG CA-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>Forward 5’-TGC TCA TAG CAG CCA CCT TC-3’</td>
<td>33</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-GTC CAT GGA ATC CTG AAC CC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL-5</td>
<td>Forward 5’-CGT GCC CAC ATC AAG GAG-3’</td>
<td>33</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-GGA CAA GAG CAA GCA GAA AC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>Forward 5’-ACC TGG ATG CTG GAG GTC T-3’</td>
<td>28</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-GCT CCA TTT GCA GCA GCT C-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p21</td>
<td>Forward 5’-AAT CTG TCA TGC TGG TCT GC-3’</td>
<td>25</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-ACT GTG ATG CGC TAA TGG C-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>Forward 5’-GGA AAG CGT AGA CAA GGA-3’</td>
<td>25</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-GGT GGG AGG GTA GAG TGG-3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Amplification products were resolved by 3% agarose gel electrophoresis, stained with SYBR green and photographed using fluorescence in LAS-4000.

**STAT3 promoter luciferase reporter gene assay**

HeLa cells were transfected with P-STAT3-TA-luc vector supplied from Clontech (Mountain View, CA, USA) with FuGENE from Promega according to supplier’s instructions. The HeLa/P-STAT3-Luc cells were seeded at a density of 8 x 10^4 per well in six-well plates. Cells were pretreated with 15-keto PGE_2 or 13,14-dihydro-15-keto
PGE₂ for 24 h and then stimulated with 10 ng/ml of Oncostatin M for 6 h. The cells were washed by PBS and lysed in 1x reporter lysis buffer from Promega. Twenty μl of lysed cell extract were mixed with 100 μl of the luciferase substrate, and the luciferase activity was determined using a luminometer (AutoLumat LB 953, EG&G Berthold). The β-galactosidase activity was measured to normalize the luciferase activity.

Wound migration assay

The Culture-Inserts (Ibidi) were transferred to 6-well plates. MCF10A and MCF10A-ras cells were seeded at a density of 5 x 10⁴ cells per well in Culture-Inserts. After 24 h, the silicon inserts were removed, and the cells were photographed under a microscope. The separated walls were closed after 24 h, and these closed gap images were captured using a microscope.

Clonogenic assay

MCF10A-ras cells were plated in 6-well plates at a density of 200 cells per well. The DMEM/F-12 medium was changed every another day and treated with 15-keto PGE₂ (20 μM) or 13,14-dihydro-15-keto PGE₂ (20 μM). After 7 days, the cells formed colonies. The colonies were fixed in cold methanol and stained by 0.5% crystal violet. The stained colonies were washed with PBS to remove excessive dye. Quantitative changes in clonogenicity were determined by extracting stained dye with 10% acetic acid and measured the absorbance of the extracted dye at 570 nm.

Anchorage independent growth assay

MCF10A-ras cells were plated on a 60 mm dish containing 0.5% (down) and 0.33%
(up) double layer agar. The dishes were incubated at 37°C in humidified incubator for 21 days. The cells were treated day after day with DMEM/F-12 containing DMSO, 15-keto PGE₂ (20 μM) or 13,14-dihydro-15-keto PGE₂ (20 μM). The colonies were stained with crystal violet for 12 h and rinsed with PBS. The number of colonies was counted by the ECLIPSE Ti inverted microscope and the NIS-Elements AR (V.4.0) computer software program (NIKON Instruments Korea, Seoul, Korea).

**Immunoprecipitation**

MCF10A-ras cells were plated in a 100 mm dish and treated with 15-keto PGE₂ (20 μM), 13,14-dihdro-15-keto PGE₂ (20 μM) or biotinylated 15-keto PGE₂ (40 μM) for indicated time. The cells were washed with ice-cold PBS and lysed in 1x lysis buffer for 1 h on ice followed by centrifugation at 18,000 g for 15 min. The protein concentration was determined by using the BCA protein assay kit (Pierce, Rockford, IL, USA). Cell lysates (500 μg) were subjected to immunoprecipitation by shaking with primary STAT3 antibody and protein A/G agarose bead suspension at 4°C for 12 h. After centrifugation at 18,000 g for 1 min, immunoprecipitation beads were collected by pouring the supernatant and washed with cell lysis buffer. The immunoprecipitation beads were then mixed with 25 μl of 2x SDS electrophoresis sample buffer and boiled for 5 min at 99°C. Supernatant (25 μl) from each sample was collected by centrifugation and loaded on SDS-polyacrylamide gel. For exogenous STAT3 homo-dimerization, PC3 cells were co-transfected with HA-tagged and Myc-tagged constitutively active STAT3s. The transfected PC3 cells were treated with 15-keto PGE₂ (20 μM) or 13,14-dihydro-15-keto PGE₂ (20 μM) for 12 h. Lysates from transfected PC3 cells were immunoprecipitated
with indicated antibodies.

**Immunocytochemistry**

MCF10A-ras cells were plated on the 8-well chamber slides at a density of $1 \times 10^4$ per well and treated with 15-keto PGE$_2$ or 13,14-dihydro-15-keto PGE$_2$. Cells were fixed in 4% formaldehyde for 15 min at 37°C. After rinse with PBST, cells were treated with 0.1% Triton X-100 in PBS for 5 min and washed with PBST. Samples were blocked with 0.05% Tween-20 in PBS containing 5% bovine serum albumin (BSA) at room temperature for 30 min and washed with PBST and then incubated with diluted (1:200) primary antibody for overnight at 4°C. After washing with PBST, samples were incubated with a diluted (1:1000) TRITC-conjugated anti-mouse secondary antibody in PBST containing 5% BSA at room temperature for 1 h. Samples were washed with 0.05% PBST containing 5% BSA then examined under a fluorescent microscope.

**Docking study**

A docking study was performed using covalent docking modules implemented in Maestro v9.5 (Schrödinger LLC, NY). The STAT3 crystal structure (PDB code: 1BG1) was retrieved from PDB bank. The protein structure and ligand were prepared according to the standard procedure of the Protein Preparation Wizard and Ligprep modules in Maestro v9.5. After adding hydrogens, STAT3 protein was neutralized and then energically minimized on only hydrogens. The structure of 15-keto PGE$_2$ was drawn by ionization at pH 7.4 and energy minimization. 15-Keto PGE$_2$ was docked to the molecular model of STAT3 *in silico*. In covalent docking module, the reaction type was set to Michael addition between Cys$^{251}$ or Cys$^{259}$ of STAT3 protein and C13 atom of 15-
keto PGE$_2$. The grid box was automatically determined the residues within 5.0 Å of the Cys$^{251}$ or Cys$^{259}$ residue in STAT3. After minimization of residues within 3 Å of covalently bound ligand, the outputs 10 poses of ligand-receptor complex was scoring by Prime MM-GBSA to calculate a binding affinity. The best binding energy of 15-keto PGE$_2$, low PrimeΔG$_{bind}$, was chosen for the binding mode analysis.

**Xenograft model**

BALB/c (nu/nu) athymic mice were purchased from Charles River Laboratories (Skokie, IL, USA) and kept in a room at constant temperature (24 ± 2°C) and humidity (50 ± 10%). The animal study was approved by Seoul National University Institutional Animal Care and Use Committees (IACUC). Seven-week old female BALB/c nude mice were subcutaneously injected with 2 x 10$^6$ MDA-MB-231 cells per mouse at both flanks. After 10 days, mice were randomly assigned to three groups (seven mice per group) and were treated with vehicle (5% DMSO in PBS), 15-keto PGE$_2$ (70 μg/kg) or 15-keto PGE$_2$ (140 μg/kg) daily by s.c. injection for 4 weeks. Tumor volumes were measured every other day with a digital caliper and calculated by the formula 0.52 x length x width$^2$. Mice were weighed three times a week.

**Hematoxylin and eosin (H&E) staining and immunohistochemical analysis (IHC) of xenograft tumors**

The MDA-MB-231 xenograft tumors were removed and fixed in formalin solution (10% neutral buffered formaldehyde) at room temperature for 48 h. Slides containing 4 μm section of formalin-fixed and paraffin-embedded specimens of xenograft tumors were prepared for histopathological and immunohistochemical analyses. H&E staining
was carried out as described previously [31]. The tumor sections were used to determine the expression of P-STAT3Y705 by immunohistochemical analysis. Slide incubated with primary antibodies for P-STAT3Y705 and developed using anti-rabbit horseradish peroxidase Envision System (DAKO). The counterstaining was done using Mayer’s hematoxylin.

Statistical analysis

Statistical analysis for single comparison was performed using the Student’s t-test. Data were expressed as means of ± SD at least three independent experiments. p < 0.05 was considered a statistically significant difference.
Results

The relationship between cellular PGE\textsubscript{2} levels and STAT3 phosphorylation in MCF10A-ras cells compared to non-oncogenic MCF10A

Arachidonic acid generates PGE\textsubscript{2} through the action of COX-2. 15-PGDH oxidizes the 15(S)-hydroxyl group of PGE\textsubscript{2} to generate 15-keto PGE\textsubscript{2} (Fig. 1A) [23, 32]. The PGE\textsubscript{2}-STAT3 axis plays a key role in cancer cell growth and metastasis in several cancer types [20-22, 33]. In human breast cancer cells, PGE\textsubscript{2} production was elevated compared to normal cells [34].

Total lysates of MCF10A and MCF10A-ras cells were analyzed for the basal levels of COX-2, 15-PGDH, STAT3 and phosphorylated STAT3. The protein level of 15-PGDH was higher in normal breast MCF10A cells compared to oncogenic MCF10A-ras cells. However, the levels of COX-2 and phosphorylated STAT3 were significantly elevated in MCF10A-ras cells (Fig. 1B). The result showed a positive correlation between COX-2 and phosphorylated STAT3 whereas there was an inverse relationship between 15-PGDH and COX-2 or P-STAT3\textsuperscript{Y705}. Moreover, the wound healing ability in MCF10A-ras cells is greater than that in non-oncogenic MCF10A cells (Fig. 1C).

Inhibitory effects of 15-Keto PGE\textsubscript{2} on STAT3 phosphorylation in MCF10A-ras cells

Phosphorylation of STAT3 at tyrosine 705 is known to be an important event for activation of this transcription factor and is implicated in cancer progression [35]. 15-Keto PGE\textsubscript{2} significantly decreased STAT3 phosphorylation in a dose- (Fig. 2A) and time-dependent manner (Fig. 2B).

PPAR\textgamma has been known to be an anticancer target [36]. Recent studies show that the oxidized prostaglandin metabolites modulate PPAR\textgamma activity as endogenous ligands of
this transcription factor [30]. 15-Keto PGE₂ is one of oxidized forms of prostaglandins and exerts antitumor effects by activating PPARγ [25, 26]. However, suppression of STAT3 phosphorylation through 15-keto PGE₂ did not appear to depend on PPARγ activity as nuclear accumulation of this transcription factor was not much different between control and 15-keto PGE₂-treated groups (Fig. 2C). Next, I investigated whether 15-keto PGE₂ could inhibit STAT3 phosphorylation through regulation of JAK2, a well-known kinase responsible for STAT3 phosphorylation [35, 37, 38], and suppressor of cytokine signaling 3 (SOCS3), a STAT3 negative regulator [39]. 15-Keto PGE₂ (20 μM) affected neither JAK2 phosphorylation nor SOCS3 expression while STAT3 phosphorylation was suppressed (Fig. 2D).

**Inhibition of STAT3 dimerization and nuclear translocation by 15-Keto PGE₂**

Phosphorylated STAT3 forms a homodimer, accumulates in the nucleus, binds to specific DNA sequences and then initiates transcription of target genes [40]. Treatment of MCF10A-ras cells with 10 or 20 μM of 15-keto PGE₂ resulted in interference of STAT3 homodimerization (Fig. 3A). As a consequence, P-STAT³Y705 barely accumulated in the nucleus of 15-keto PGE₂-treated cells (Fig. 3B and C).

**15-Keto PGE₂-mediated inhibition of MCF10A-ras cell proliferation and growth through suppression of transcription of STAT3 target genes**

Dimerized STAT3 localizes in the nucleus [14], where it regulates the expression of target genes. To assess the effect of 15-keto PGE₂ on transcriptional activity of STAT3, I performed a luciferase reporter gene assay. 15-Keto PGE₂ inhibited the Oncostatin M
(OSM)-induced STAT3 transcriptional activity in HeLa/P-STAT3-Luc cells (Fig. 4A).

Nuclear translocated STAT3 binds to the promoters of numerous target genes [41]. Some of the protein products of STAT3 target genes have oncogenic functions [42]. These include monocyte chemoattractant protein-1 (MCP-1), chemokine (C-C motif) ligand 5 (CCL5), cyclin D1, Bcl-xL, p21WAF/Cip1 (cyclin-dependent kinase inhibitor 1) and myeloid cell leukemia 1 (MCL-1). 15-Keto PGE2 modulated the expression of these STAT3 target genes (Fig. 4B). The clonogenic assay and the anchorage-independent growth assay were conducted to investigate anti-proliferative effects of 15-keto PGE2. Results show that 15-keto PGE2 treatment markedly reduced the colony formation (Fig. 4C and D).

The involvement of the α,β-unsaturated carbonyl moiety of 15-Keto PGE2 in STAT3 deactivation

15-Keto PGE2 has an α,β-unsaturated carbon which is capable of interacting with nucleophilic cellular proteins [26]. In contrast to 15-keto PGE2, 13,14-dihydro-15-keto PGE2 that lacks an electrophilic α,β-unsaturated carbonyl group (Fig. 5A) failed to inhibit STAT3 phosphorylation (Fig. 5B). In addition, 13,14-dihydro-15-keto PGE2-treated MCF10A-ras cells did not exhibit a significant change in homodimerization (Fig. 5C). PC3 cells were transfected with HA-tagged STAT3 and Myc-tagged STAT3 followed by treatment with 15-keto PGE2 (20 μM) or 13,14-dihydro-15-keto PGE2 (20 μM), and the immunoprecipitation assay was conducted. The STAT3 dimerization was inhibited by 15-keto PGE2 but not by 13,14-dihydro-15-keto PGE2 (Fig. 5D). Likewise, the nuclear accumulation of phosphorylated STAT3 was not attenuated by 13,14-dihydro-15-keto PGE2 as well (Fig. 5E and F). Furthermore, the transcriptional activity
of STAT3 was again not affected by 13,14-dihydro-15-keto PGE₂ (Fig. 5G). The colony formation was suppressed by 15-keto PGE₂ but not by 13,14-dihydro-15-keto PGE₂ (Fig. 5H and I). Above results suggest that the electrophilic α,β-unsaturated carbonyl group of 15-keto PGE₂ is important for deactivating STAT3.

**Covalent binding of 15-Keto PGE₂ to STAT3 via Michael addition reaction**

The electron-poor-β-carbon in the α,β-unsaturated carbonyl group attacks electron-rich nucleophiles, forming a covalent bond via Michael addition reaction [43, 44]. As shown in Fig. 6A, 15-keto PGE₂ harboring the electrophilic α,β-unsaturated carbonyl moiety is supposed to covalently bind to thiol group(s) of STAT3. To investigate a putative covalent interaction between 15-keto PGE₂ and a thiol group of STAT3, MCF10A-ras cells were pretreated with thiol reducing agents, such as DTT and NAC. The inhibition of STAT3 phosphorylation by 15-keto PGE₂ was abolished by DTT or NAC treatment (Fig. 6B). To investigate direct interaction between 15-keto PGE₂ and STAT3, an immunoprecipitation assay using the biotin-streptavidin system was performed. The result showed that biotinylated 15-keto PGE₂ directly bound to STAT3 (Fig. 6C). However, DTT and NAC abrogated the interaction between 15-keto PGE₂ and STAT3 (Fig. 6D). A docking model predicted that Cys²⁵¹ and Cys²⁵⁹ of STAT3 as putative binding sites of 15-keto PGE₂ (Fig. 6E). Taken together, these data suggest that 15-keto PGE₂ covalently interacts with STAT3 through thiol modification of this transcription factor.
Suppression of tumor growth and STAT3 phosphorylation by 15-Keto PGE₂ in a MDA-MB-231 xenograft model

When human breast cancer MDA-MB-231 cells were treated with 10 or 20 μM of 15-keto PGE₂, colony formation was repressed (Fig. 7A). The expression of phosphorylated STAT3 was significantly decreased by 15-keto PGE₂ in MDA-MB-231 cells (Fig. 7B). I examined whether 15-keto PGE₂ has anti-tumor activity in MDA-MB-231 cells transplanted to BALB/c nude mice. The mice xenografted with MDB-MB-231 cells were subcutaneously injected with two different doses (70 μg/kg and 140 μg/kg) of 15-keto PGE₂ for 4 weeks. The tumor growth was significantly retarded in 15-keto PGE₂-injected mice groups (Fig. 7C and D). The average tumor volume in 15-keto PGE₂-treated mice was dramatically reduced in a dose-dependent manner (Fig. 7E). Under these experimental conditions, there were no body weight loss and other signs of toxicity in mice treated with 15-keto PGE₂ (Fig. 7F). Histopathological analysis of the tumor samples stained for H&E showed that tumor density was decreased in 15-keto PGE₂-treated mice compared to that in the control animals (Fig. 7G). Furthermore, the phosphorylation of STAT3 was markedly decreased in the group treated with the higher dose (140 μg/kg) of 15-keto PGE₂ (Fig. 7H). Immunohistochemical analysis of tumor samples showed that the expression of P-STAT3 Y705 was significantly reduced in the group treated with the higher dose (140 μg/kg) of 15-keto PGE₂, supporting the western blotting data (Fig. 7I).
Figure 1. The relationship between cellular PGE\textsubscript{2} levels and STAT3 phosphorylation in MCF10A-ras cells compared to non-oncogenic MCF10A A. A scheme of PGE\textsubscript{2} synthesis and degradation. PGE\textsubscript{2} is synthesized by COX-2 and degraded by 15-PGDH. 15-Keto PGE\textsubscript{2} is an oxidized metabolite of PGE\textsubscript{2}. B. The
protein levels of 15-PGDH, COX-2, STAT3 and P-STAT3Y705 were compared in non-oncogenic MCF10A and MCF10A-ras cells by Western blot analysis. Actin was used as a loading control. C. The migration of MCF10A and MCF10A-ras cells was assessed by measuring the width of un-migrated space under a microscope.
Figure 2. Inhibitory effects of 15-Keto PGE$_2$ on STAT3 phosphorylation in MCF10A-\textit{ras} cells. A. MCF10A-\textit{ras} cells were treated with indicated concentrations of 15-keto PGE$_2$ for 24 h. The expression levels of STAT3 and P-STAT3$^{Y705}$ were
determined by Western blot analysis. B. MCF10A-ras cells were incubated with 15-keto PGE₂ (20 μM) for indicated time periods. The protein level of phosphorylated STAT3 was measured by Western blotting. C. Nuclear extracts were isolated from 15-keto PGE₂ (20 μM)-treated MCF10A-ras cells, and nuclear PPARγ levels were analyzed by Western blot analysis. D. MCF10A-ras cells were treated with 15-keto PGE₂ (20 μM) for 24 h. The expression of JAK2, an upstream kinase of STAT3 and SOCS3, a negative regulator of STAT3, were assessed by Western blot analysis.
Figure 3. Inhibition of STAT3 dimerization and nuclear translocation by 15-Keto PGE₂

A. Whole lysates from MCF10A-ras cells treated with two different concentrations of 15-keto PGE₂ (10 or 20 μM) for 12 h were prepared for immunoprecipitation of STAT3 protein using protein A/G agarose conjugated C-terminus anti-STAT3 antibody. The STAT3 homodimerization was analyzed by Western blot analysis using N-terminus anti-STAT3 antibody. B. Nuclear extracts were immunoblotted for detecting P-STAT3 Y705 localization. MCF10A-ras cells were treated with 15-keto PGE₂ (20 μM). After 24 h, the cells were harvested. Lamin B was used as loading control in nuclear extract. C. Immunocytochemical analysis was performed...
using antibodies against P-STAT3 

MCF10A-ras cells were treated with or without 15-keto PGE₂ (20 μM) for 24 h. Nuclei were stained with DAPI and visualized under a confocal microscope. Two images were merged to verify co-localization.
Figure 4. 15-Keto PGE$_2$-mediated inhibition of MCF10A-ras cell proliferation and growth through suppression of transcription of STAT3 target genes. A. The luciferase assay was performed with HeLa/P-STAT3-Luc reporter cells. The cells were
treated with 15-keto PGE$_2$ (10 or 20 μM) for 24 h and then stimulated with Oncostatin M (10 ng/ml) for another 6 h. Cells were then analyzed by the microplate luminometer.

B. The mRNA and protein levels of STAT3 target genes were determined by RT-PCR and Western blot analyses, respectively. MCF10A-ras cells were treated with 15-keto PGE$_2$ (20 μM) or vehicle for 24 h. GAPDH gene expression was measured to ensure equal amount of cDNA loaded. C and D. The clonogenic assay and the anchorage-independent growth assay were performed in MCF10A-ras cells. MCF10A-ras cells were seeded in 6 well plates and treated with two different concentrations of 15-keto PGE$_2$ (10 or 20 μM) or vehicle (DMSO) in 1 ml DMEM/F12 medium containing 5% heat-inactivated horse serum and supplementary agents. The soft agar colonies were counted under a light microscope. (colony size > 100 μm) For quantitative clonogenic assays, the colonies were solubilized with 10% acetic acid, and the absorbance at the wavelength of 540 nm was read. Results are the means ± S.D of triplicate experiments.
Figure 5. The involvement of the $\alpha,\beta$-unsaturated carbonyl moiety of 15-Keto
**PGE2 in STAT3 deactivation**

A. 15-Keto PGE2 has an α,β-unsaturated carbon which is considered to target nucleophiles whereas 13,14-dihydro-15-keto PGE2, a metabolite of 15-keto PGE2, has no such electrophilic moiety.

B. MCF10A-ras cells were treated with 15-keto PGE2 (20 μM) or 13,14-dihydro-15-keto PGE2 (20 μM) for indicated time periods. The expression levels of P-STAT3<sup>Y705</sup> and STAT3 were measured by Western blot analysis.

C. Homodimerization of STAT3 was measured by the immunoprecipitation assay. MCF10A-ras cells were treated with 15-keto PGE2 (20 μM) or 13,14-dihydro-15-keto PGE2 (20 μM) for 12 h.

D. Human prostate cancer PC3 cells were co-transfected with HA-tagged STAT3 and Myc-tagged STAT3 and treated with 15-keto PGE2 or 13,14-dihydro-15-keto PGE2 (20 μM each) for 12 h. The total lysates obtained from the transfected cells were immunoprecipitated with anti-Myc antibody and analyzed by Western blotting with anti-HA antibody.

E. MCF10A-ras cells were incubated with 15-keto PGE2 (20 μM) or 13,14-dihydro-15-keto PGE2 (20 μM) for 24 h. The nuclear extracts were separated and subjected to Western blot analysis. F. After MCF10A-ras cells were treated with 15-keto PGE2 (20 μM) or 13,14-dihydro-15-keto PGE2 (20 μM) for 24 h, immunocytochemical analysis of P-STAT3<sup>Y705</sup> was conducted.

P-STAT3<sup>Y705</sup> was detected using TRITC red fluorescence-labeled secondary antibody.

G. The HeLa/P-STAT3-Luc reporter cells were pretreated with 15-keto PGE2 (20 μM) or 13,14-dihydro-15-keto PGE2 (20 μM) for 24 h followed by treatment with oncostatin M (OSM) for another 6 h. The cells were analyzed for the luciferase activity using a microplate luminometer.

H and I. MCF10A-ras cells treated with 15-keto PGE2 (20 μM) or 13,14-dihydro-15-keto PGE2 (20 μM) were subjected to the clonogenic assay and the anchorage independent growth assay. The soft agar colonies were counted under a light microscope. (colony size > 100 μm) For quantitative clonogenic assays, the colonies...
were solubilized with 10% acetic acid, and the absorbance at 540 nm was read. Results are the means ± S.D of triplicate experiments.
Figure 6. Covalent binding of 15-Keto PGE₂ to STAT3 via Michael addition reaction A. A proposed interaction between 15-keto PGE₂ and a thiol group of STAT3. B. MCF10A-ras cells were pretreated with thiol a reducing agent, DTT (100 μM) or NAC (5 mM), for 1 h followed by exposure to 15-keto PGE₂ (20 μM) for an additional
24 h. The protein levels of P-STAT3<sup>Y705</sup> and STAT3 were determined by Western blot analysis. C. The direct binding of 15-keto PGE<sub>2</sub> to STAT3 was assessed using the biotin-streptavidin system. MCF10A-ras cells were exposed to biotinylated 15-keto PGE<sub>2</sub> (40 μM) for 24 h. The biotinylated 15-keto PGE<sub>2</sub>-STAT3 complex was precipitated by monoclonal STAT3 antibody followed by immunoblot analysis with streptavidin-horse radish peroxidase secondary antibody. D. MCF10A-ras cells were pretreated with DTT (100 μM) or NAC (5 mM), for 1 h and then treated with biotinylated 15-keto PGE<sub>2</sub> (40 μM) for an additional 12 h. The biotinylated 15-keto PGE<sub>2</sub>-STAT3 complex formation was determined by the immunoprecipitation and Western blot analyses. E. A putative model of 15-keto PGE<sub>2</sub> covalently binding to Cys<sup>259</sup> and/or Cys<sup>251</sup> of STAT3 as predicted by a docking study.
Figure 7. Suppression of tumor growth and STAT3 phosphorylation by 15-Keto
**PGE$_2$ in a MDA-MB-231 xenograft model**  
A. 15-keto PGE$_2$ (20 μM) treated MDA-MB-231 cells were subjected to the clonogenic assay.  
B. MDA-MB-231 cells were treated with 15-keto PGE$_2$ (20 μM) for 48 h. The expression levels of P-STAT$^Y705$ and STAT3 were measured by Western blot analysis.  
C. The representative shots of human mammary tumor (MDA-MB-231) xenografts in mice treated with vehicle, 15-keto PGE$_2$ (70 μg/kg) or 15-keto PGE$_2$ (140 μg/kg) for 4 weeks ($n = 14$/group).  
D. The size of tumors was measured with digital calipers. The calculated formula is $0.52 \times \text{length} \times \text{width}^2$. ** $p < 0.005$, *** $p < 0.001$ (Two-sided t-test).  
G. H&E stained tumor tissue sections. Scale bar: 100 μM. Magnification: x 4 and x 10.  
H. Total protein samples from the tumor specimens were subjected to measurement of the levels of P-STAT$^Y705$ and STAT3 by Western blot analysis. Actin was used as the internal control.  
I. The expression of P-STAT$^Y705$ was determined by immunohistochemical analysis in the tumor sections. Scale bar: 100 μM. Magnification: x 10
**Discussion**

PGE$_2$ is involved in chronic inflammation and it contributes to cancer development [3, 5]. PGE$_2$ and its receptors (EP1, EP2, EP3 and EP4) play important roles in promoting cancer progression [20-22, 33, 45]. PGE$_2$ exerts its function through EP receptors which are coupled to different signaling pathways [46-48]. STAT3 signaling was found to be inhibited by an EP receptor antagonist, which resulted in suppression of hepatocellular carcinoma cell growth [49]. As downstream effectors of PGE$_2$, activated EP receptors are involved in cancer progression. In mammary tumors, two subtypes of EP receptors, EP2 and EP4 are overexpressed. One study showed that mammary tumor angiogenesis and progression were suppressed through downregulation of EP2 and EP4 by a COX-2 inhibitor [47]. PGE$_2$ is rapidly metabolized to 15-keto PGE$_2$ [50]. A recent study suggests that 15-keto PGE$_2$ contributes to termination or attenuation of EP2 and EP4 responses [51]. Thus, 15-keto PGE$_2$ appears to exert anti-cancer effects through interruption of coupling PGE$_2$ and EP receptors. However, I found that 15-keto PGE$_2$ directly bound to STAT3. This suggests that the tumor suppressive effects of this lipid mediator may also arise from an EP receptor-independent mechanism.

Numerous human cancers harbor persistently activated STAT3 which induces tumor formation, growth and survival [13, 16]. Accumulating evidence indicates that PGE$_2$ has a close association with the STAT3 signaling pathway [20-22, 49]. JAK2 is one of STAT3 upstream kinases, and the JAK2/STAT3 pathway has been extensively investigated in breast and other cancer types [52]. Inhibition of JAK2 activity deactivates STAT3 in most breast cancer cells [53]. Given the importance of the JAK2/STAT3 axis in cancer, inhibiting this kinase seems to be a reasonable approach.
However, a recent study suggested that a JAK2 inhibitor, cucurbitacin I, also affected the actin cytoskeleton in non-tumor cells [54]. Furthermore, some studies demonstrated that JAK2 inhibits tumor initiation as a tumor suppressor [55-57]. Therefore, direct inhibition of STAT3 could be a more effective strategy to inhibit cancer progression. Interestingly, I observed that 15-keto PGE$_2$ selectively inhibited STAT3 phosphorylation without affecting the JAK2 activity or the SOCS3 expression.

PPAR$\gamma$ is a member of nuclear receptor superfamily, which functions as ligand-dependent activators of transcription [58]. PPAR$\gamma$ ligands have been shown to inhibit cell growth and induce apoptosis in several types of human cancer, suggesting that this transcription factor may be a tumor suppressor gene [36]. One study showed that an endogenous PPAR$\gamma$ ligand, 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$ (15d-PGJ$_2$) effectively blocked DNA binding and transcriptional activity of STAT3 through activation of PPAR$\gamma$ [59]. I attempted to investigate whether the inhibitory effect of 15-keto PGE$_2$ on STAT3 activation is mediated by PPAR$\gamma$. 15-Keto PGE$_2$ inhibits STAT3 signaling in MCF10A-ras cells, which was unlikely to be dependent on PPAR$\gamma$ activation. This suggests that there may be an alternative mechanism for blocking STAT3 signaling by 15-keto PGE$_2$. 15-Keto PGE$_2$ has an electrophilic $\alpha,\beta$-unsaturated carbonyl group that can covalently modify nucleophilic cysteine residue(s) present in various proteins, regulating their functions/activities [30, 60, 61]. In the present study, 15-keto PGE$_2$ formed an adduct with thiol group(s) of cysteine residue(s) in STAT3. STAT3 signaling was inhibited by 15-keto PGE$_2$ but not by 13,14-dihydro-15-keto PGE$_2$ which lacks the $\alpha,\beta$-unsaturated carbonyl group. The direct interaction between 15-keto PGE$_2$ and STAT3 was disrupted by thiol reducing agents, such as DTT or NAC. Above results suggest that the Michael addition reaction occurs between the $\alpha,\beta$-unsaturated carbonyl
group of 15-keto PGE₂ and cysteine residue(s) in STAT3.

STAT3 consists of 6 domains. These include N-terminal domain (ND), a coiled-coil domain (CCD), a DNA binding domain (DBD), a linker domain (LK), Src-homology 2 (SH2) domain and C-terminal transactivation domain. Recent studies suggest that alkylation of a cysteine residue in STAT3 is a plausible mechanism of STAT3 inhibition [62, 63]. A small molecule compound, NSC-368262 (C48) alkylates Cys⁴⁶⁸ in DNA binding domain of STAT3, thereby suppressing DNA binding of this transcription factor [64]. A docking study predicted Cys²⁵¹ and Cys²⁵⁹ in STAT3 as preferential targets for a covalent linkage formation with 15-keto PGE₂. These two cysteine residues are located in the coiled-coil domain of STAT3. It is well known that the SH2 domain of STAT3 is essential for binding to an activated receptor, gp130 and that STAT3 is phosphorylated by the gp130-associated JAKs [65, 66]. However, one study demonstrated that the coiled-coil domain also interacts directly with the gp130 peptide and is required for STAT3 phosphorylation [66]. It is therefore plausible that 15-keto PGE₂ inhibits STAT3 phosphorylation by binding to this domain. It still remains to be investigated which cysteine residue(s) is/are likely to be the site(s) for 15-keto PGE₂ to target.

The tumor suppressive potential of 15-keto PGE₂ was evaluated in human mammary tumor xenograft model. The growth of MDA-MB-231 xenograft tumors was suppressed by subcutaneous treatment of 15-keto PGE₂ without significant side effects. Furthermore, STAT3 phosphorylation was significantly inhibited in the high dose-treated group. In conclusion, 15-keto PGE₂ inhibits STAT3 signaling by covalently modifying this protein, thereby suppressing breast cancer proliferation and growth.
References

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국문초록

Prostaglandin E₂ (PGE₂)는 염증반응과 암화과정에서 대량으로 생성된다. 과발현한 PGE₂는 암세포의 증식과 암세포사멸 저항성, 암세포 전이와 관련이 있다. PGE₂는 15-hydroxyprostaglandin dehydrogenase (15-PGDH)에 의해 산화되어 15-keto prostaglandin E₂ (15-keto PGE₂)로 전환된다. 15-keto PGE₂는 이때까지 PGE₂의 생리불활성 형태로서 알려져 있다. 그러나, 최근 연구에 따르면 15-keto PGE₂는 여러 종양활성신호를 차단함으로써 종양 억제 효과가 있음을 입증되었다. 그러나 이에 따른 분자적 기전연구에 대해서는 연구가 많이 되어있지 않다.

Signal transducer and activator of transcription factor 3 (STAT3)는 전사인자로 세포 증식을 유도하면서 동시에 세포 사멸 저항성을 가지고 있다. 정상세포와는 다르게 많은 암세포에서 STAT3는 활성화 상태를 계속 유지하고 있다. 지속적으로 활성화된 STAT3는 발암과 암화과정에서 주요한 역할을 한다. 이러한 STAT3 신호전달과정이 15-PGDH에 의하여 억제된다는 보고가 있지만 그에 따른 분자적 기전연구에 대해서는 이루어지지 않았다. 따라서 본 연구에서는 15-PGDH에 의해 생성된 대사물질인 15-keto PGE₂가 STAT3 불활성화 효과가 있는지 확인하였다.


이러한 전자성을 띄는 15-keto PGE₂의 α,β 불포화 카보닐기가 STAT3 시스템 전기의 티올기와 결합하여 STAT3 활성 억제 가능성을 확인하였다. 15-keto PGE₂는 STAT3와 직접적인 결합을 하고, 그에 따라 STAT3 불활성을 유도함을 확인하였지만, 티올기 환원체로 알려져 있는 dithiothreitol (DTT) 또는 N-acetyl-L-cysteine (NAC)을 처리하였을 때 15-keto PGE₂에 의한 STAT3 불활성화와 서로 간의 직접적인 결합은
이루어지지 않았다. Computer docking modeling에서 15-keto PGE₂가 STAT3 시스템 잔기 251번과 259번에 직접 결합 가능성을 제시하였다.


주요어: 15-Keto PGE₂, 천전자성, α, β 불포화 카보닐기, STAT3, 타올기 변화, MCF10A-ras, MDA-MB-231 동물 모델

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