15-Keto prostaglandin E$_2$ induces heme oxygenase-1 expression through activation of Nrf2 in human colon epithelial CCD 841 CoN cells

Jeong-Eun Lee[a,1], Xiancai Zhong[b,1], Ja-Young Lee[c], Young-Joon Surh[b,d], Hye-Kyung Na[a,*]

[a] Department of Food Science and Biotechnology, College of Knowledge-Based Services Engineering, Sungshin Women's University, Seoul 01133, Republic of Korea
[b] Tumor Microenvironment Global Core Research Center, College of Pharmacy, Seoul National University, Seoul 08826, Republic of Korea
[c] Department of Food and Nutrition, College of Health & Wellness, Sungshin Women's University, Seoul 01133, Republic of Korea
[d] Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science and Technology, Seoul National University, Seoul 08826, Republic of Korea

ARTICLE INFO

ABSTRACT

Prostaglandin E$_2$ (PGE$_2$) plays a key role in inflammation-associated carcinogenesis. NAD$^+$-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH) catalyzes the oxidation of the 15(S)-hydroxyl group of PGE$_2$ to generate 15-keto PGE$_2$. 15-PGDH has been known as a tumor suppressor in various malignancies including colon cancer. However, the molecular mechanisms underlying the tumor-suppressive function of 15-PGDH remain largely unresolved. In this study, we found that 15-keto PGE$_2$ upregulated the expression of heme oxygenase-1 (HO-1), a representative antioxidative and anti-inflammatory enzyme, at both transcriptional and translational levels, in human colon epithelial CCD 841 CoN cells. A redox-sensitive transcription factor, NF-E2-related factor (Nrf2) plays a critical role in the regulation of HO-1 and other cytoprotective proteins. 15-Keto PGE$_2$ induced translocation of Nrf2 into the nucleus and antioxidant response element-driven luciferase activity. Furthermore, the silencing of the Nrf2 gene abolished 15-keto PGE$_2$-induced HO-1 expression in CCD 841 CoN cells. 15-Keto PGE$_2$ activated AKT signaling, and the pharmacological AKT inhibitor, LY294002 suppressed the 15-keto PGE$_2$-induced HO-1 expression. 15-Keto PGE$_2$ generates the reactive oxygen species which is suppressed by the general antioxidant N-acetyl-L-cysteine. N-acetyl-L-cysteine treatment attenuated the 15-keto PGE$_2$-induced phosphorylation of GSK3β, transcriptional activity of Nrf2, and subsequently HO-1 expression. However, 13,14-dihydro-15-keto PGE$_2$ lacking the α,β-unsaturated carbonyl moiety failed to induce intracellular production of reactive oxygen species, HO-1 expression and nuclear translocation of Nrf2. In conclusion, 15-keto PGE$_2$ induces HO-1 expression through Nrf2 activation in human colon epithelial cells.

1. Introduction

Inflammatory bowel disease (IBD), such as Crohn's disease and ulcerative colitis, has been characterized as sustained chronic inflammation of small intestine and colon, which promotes the colorectal carcinogenesis (CRC). There is a vicious cycle between oxidative stress and inflammation, and oxidative stress is hence one of the pathogenic factors of IBD [1]. Overexpression of cyclooxygenase-2 (COX-2) is one of the hallmarks of IBD [2] and inflammation-associated CRC [3]. As a consequence of overexpression of COX-2, prostaglandin E$_2$ (PGE$_2$) is overproduced by the microsomal prostaglandin E synthase from the substrate, prostaglandin H$_2$, a metabolic product of arachidonic acid [4].

The intracellular level of PGE$_2$ is regulated not only by COX-2-catalyzed synthesis but also by further metabolism. 15-hydroxyprostaglandin dehydrogenase (15-PGDH) is the NAD$^+$-dependent enzyme responsible for the oxidation of PGE$_2$ to generate the 15-keto PGE$_2$. A high level of PGE$_2$ is observed in polyps and carcinoma tissue of patients [5]. 15-PGDH was found to be down-regulated in the IBD patients, indicative of the additional mechanism on a high level of PGE$_2$ in these patients [6]. Down-regulation of 15-PGDH is also observed in colon cancer and other tumors [7–11]. In contrast, the induction of 15-PGDH expression by some chemopreventive agents has been shown to suppress CRC [12,13]. For example, synthetic triterpenoids suppress the colon carcinogenesis induced by azoxymethane plus dextran sulfate sodium (DSS) through upregulation of 15-PGDH [13]. Therefore, the induction of 15-PGDH expression is considered as a novel cancer chemopreventive strategy [14].

Abbreviations

AKT	protein kinase B	MAPK	mitogen-activated protein kinase
ARE	antioxidant response element	NAC	N-acetyl-L-cysteine
COX-2	cyclooxygenase-2	NQO-1	NAD(P)H quinone oxidoreductase
DCF-DA	2′,7′-dichlorofluorescein	Nrf2	nuclear factor-erythroid-2-related factor 2
DTT	dithiothreitol	PARP-1	poly(ADP-ribose) polymerase 1
DSS	dextran sulfate sodium	15-PGDH	15-hydroxyprostaglandin dehydrogenase
ERK	extracellular signal-regulated kinase	PGE_2	prostaglandin E_2
HO-1	heme oxygenase-1	PTEN	phosphatase and tensin homolog
JNK	c-Jun N-terminal kinase	PTGR2	prostaglandin reductase 2
Keap1	Kelch-like ECH-associated protein 1	STAT3	signal transducer and activator of transcription 3
		TXN	thioredoxin

The anti-carcinogenic and anti-inflammatory effects of 15-PGDH have been known to be mediated through its catalytic product, 15-keto PGE_2. 15-Keto PGE_2 has been shown to protect against experimentally induced sepsis [15]. It increased the p21 expression via peroxisome proliferator-activated receptor γ activation, thereby suppressing the growth of hepatocellular carcinoma [16]. In addition, 15-keto PGE_2 induces apoptosis in pancreatic cancer by suppressing the expression of solute carrier family 7 member 11 and cystathionine γ-lyase which are associated with generation of glutathione (GSH) [17]. Our recent study has demonstrated that 15-keto PGE_2 directly interacts with Cys^{259} of signal transducer and activator of transcription 3 (STAT3), leading to functional inactivation of this transcription factor [18]. We have also observed that 15-keto PGE_2 inhibits tumor growth in xenograft mice transplanted with breast cancer cells [18].

The redox-sensitive nuclear factor-erythroid-2-related factor 2 (Nrf2), a member of the basic leucine zipper transcription factors, is the guardian of redox homeostasis. It plays a pivotal role in cellular defense against not only oxidative but also inflammatory stress [19]. Nrf2 regulates the expression of antioxidant enzymes and other cytoprotective proteins to protect the cells against oxidative, electrophilic and inflammatory stresses [20]. Under the normal physiological condition, Nrf2 is degraded by its repressor Kelch-like ECH-associated protein 1 (Keap1) through the ubiquitination-proteasome pathway [21]. Under the oxidative stress, however, the release of Nrf2 from the Keap1 repression renders Nrf2 stabilized [22,23]. Accumulated Nrf2 then translocates into the nucleus, where it binds to antioxidant/electrophile response elements (ARE/EpRE) and regulates antioxidant enzymes such as NAD(P)H:quinone oxidoreductase-1 (NQO1), heme oxygenase-1 (HO-1), thioredoxin (TXN), glutathione *S*-transferase, glutathione peroxidase, superoxide dismutase, glutamate-cysteine ligase, catalase, etc. [22,23]. Nrf2 is released when it is phosphorylated by a protein kinase or Keap1 is modified in its cysteine thiol group(s) by reactive oxygen/nitrogen species or electrophiles. Because of the α,β-unsaturated carbonyl moiety 15-keto PGE_2 carries, this electrophilic lipid mediator acts as a Michael reaction acceptor, capable of reacting with diverse signaling molecules, such as Keap1, harboring nucleophilic cysteine residues.

In this study, we have attempted to determine whether 15-keto PGE_2 can activate Nrf2 signaling in normal colon epithelial cells.

2. Methods

2.1. Chemicals and reagents

15-Keto PGE_2 and 13,14-dihydro-15-keto PGE_2 were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Recombinant human interleukin (IL)-6 was purchased from R&D Systems (Minneapolis, MN, USA). N-Acetyl-L-cystein (NAC) was obtained from Sigma-Aldrich (St Louis, MO, USA). Minimum essential medium (MEM) and penicillin-streptomycin were obtained from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from HyClone (South Logan,

UT, USA). HO-1 polyclonal antibody was a product of Enzo Life Science (Postfach, Lausen, Switzerland). Primary antibodies against Nrf2, TXN, protein kinase B (AKT), phospho-AKT, c-Jun N-terminal kinase (JNK), phospho-JNK, extracellular signal-regulated kinase (ERK), phospho-ERK, p38, phospho-p38, STAT3, and phospho-STAT3 were products of Cell Signaling Technology (Beverly, MA, USA). Anti-GAPDH, anti-Lamin B, and anti-NQO1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-rabbit and anti-mouse horseradish peroxidase-secondary antibody were provided by Thermo Scientific (Rockford, MD, USA). The ECL chemiluminescent detection kit was obtained from ELPIS-BIOTECH (Daejeon, Korea). LY294002 was purchased from TOCRIS (Ellisville, MO, USA). SB203580, SP600125, and U0126 were the products of Calbiochem (San Diego, CA, USA). 2′,7′-Dichlorofluorescein diacetate (DCF-DA) was obtained from Invitrogen (Invitrogen, Carlsbad, CA, USA). Hank's balanced salt solution (HBSS) was purchased from Gibco BRL (Grand Island, NY, USA). Polyvinylidene difluoride (PVDF) membranes were supplied from Millipore (Burlington, MS, USA).

2.2. Cell culture

Human CCD 841 CoN colon normal epithelial cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were cultured in MEM supplemented with 10% (v/v) FBS, 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C in an incubator with a humidified atmosphere of 95% O_2/5% CO_2.

2.3. Nuclear and cytoplasmic protein extraction

Cytoplasmic proteins were separated in buffer A [10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1% NP-40]. Nuclear pellets were washed twice with buffer A with protease inhibitors and then lysed in buffer C [20 mM HEPES, 100% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF]. Quantification of protein was performed by the Bradford method according to standard protocols.

2.4. Western blot analysis

Cells were collected and lysed in ice-cold lysis buffer [20 mM Tris-HCL, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, and 1 μg/mL leupeptin] containing protease inhibitors (Cell Signaling Technology; Danvers, MA, USA). Lysates were centrifuged at 13,000 rpm for 15 min at 4 °C. The protein concentration was determined by using the Bio-Rad Protein Assay (Bio-Rad; Hercules, CA, USA), and 25–30 μg of protein was electrophoresed in SDS-acrylamide gels and transferred to Immobilon®-P PVDF membrane (EMD Millipore Cooperation; Billerica, MA, USA) at 100 mA for 1 h. The membrane was blocked in 5% non-fat dry milk solved in 0.05% Tween 20 in PBS

(PBST) for 1 h. Afterwards, the blots were incubated with the primary antibody diluted in PBST buffer overnight at 4 °C. After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibody. The immunoblot with protein-antibody complexes were detected by using an enhanced chemiluminescence detection kit (ELPIS-BIOTECH; Daejeon, Korea). The band was detected by the Chemidoc imaging system (CAN ICES-3, Bio-rad).

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from CCD 841 CoN colon normal epithelial cells using the easy-BLUE method (iNtRON; Burlington, MA, USA) following the manufacturer's instructions. To generate complementary DNA (cDNA), 1 μg of total RNA was reverse transcribed by using murine leukemia virus reverse transcriptase (Promega; Madison, WI, USA). Amplification of genes was performed by PCR analysis using SolgTM 2x Taq PCR Smart Mix (Solgent; Daejeon, Korea) according to

manufacturer's instructions. The primers used were as follows: *GAPDH* (Forward); 5′-AAG GTC GGA AAC GGA TTT-3′, *GADPH* (Reverse); 5′-GCA GTG AGG GTC TCT TCT CT-3′, *HMOX1* (Forward); 5′-CAG GCA GAG AAT GCT GAG TTC-3′, *HMOX1* (Reverse) 5′-GAT GTT GAG CAG GAA CGC AGT-3′, *NQO1* (Forward); 5′-CAC ACT CCA GCA GAC GCC CG-3′, *NQO1* (Reverse); 5′-TGC CCA AGT GAT GGC CCA CAG-3′, *TXN* (Forward); 5′-CAC ACT CCA GCA GAC GCC CG-3′, *TXN* (Reverse); 5′-TGC CCA AGT GAT GGC CCA CAG-3′. The amplified transcript level of each specific gene was normalized to that of *GAPDH*. The PCR product was run on 1.5% agarose gel followed by staining of DNA using RedSafe™ (iNtRON, Burlington, MA, USA). The image analysis was performed using the Chemidoc imaging system (CAN ICES-3, Bio-rad).

2.6. Immunocytochemistry

CCD 841 CoN cells were plated on the 8-well chamber slides at a density of 1×10^4 and treated with 15-keto PGE$_2$ or vehicle alone.
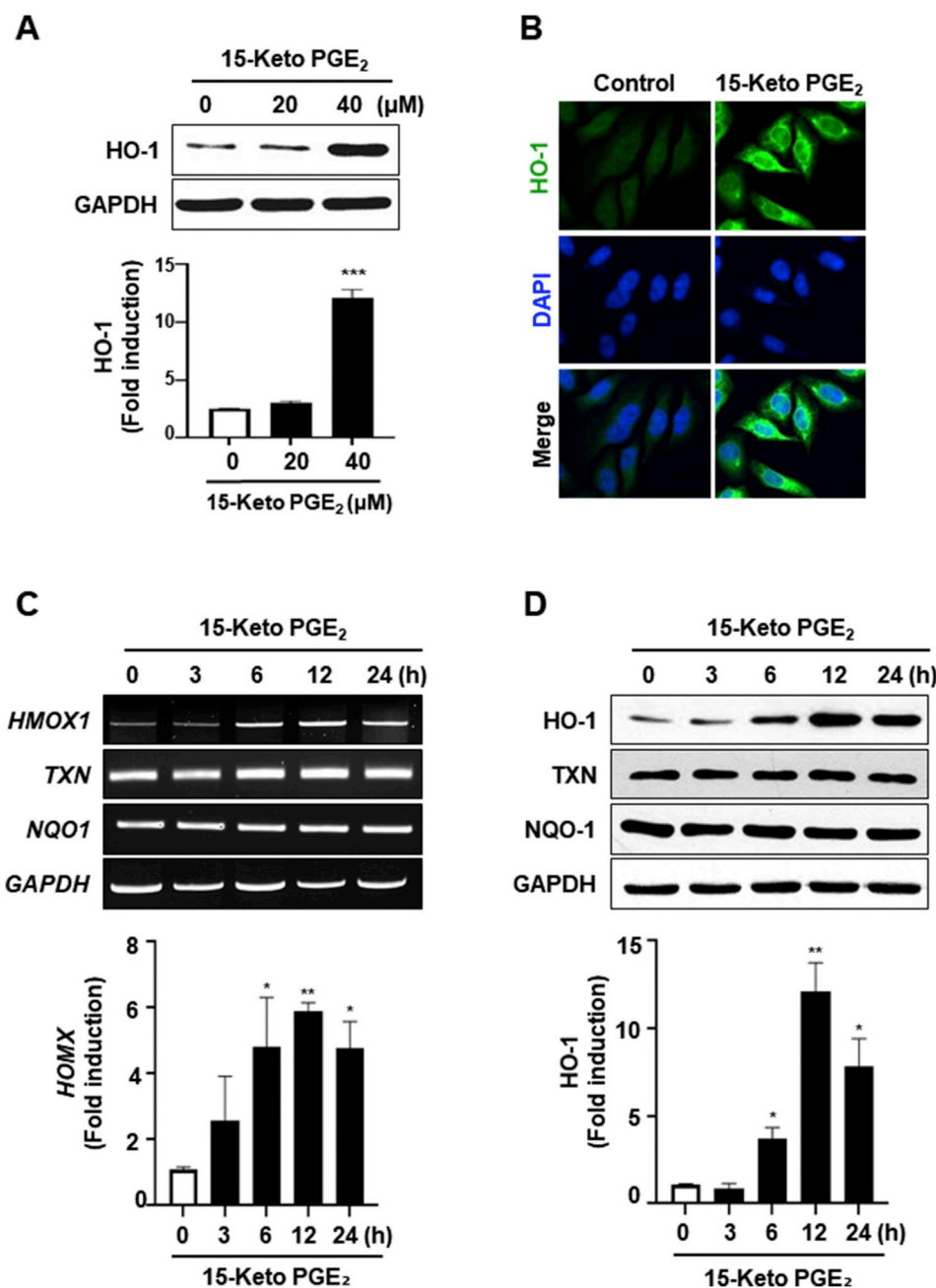

Fig. 1. 15-Keto PGE$_2$ induced expression of HO-1 in human colon epithelial CCD 841 CoN cells. (A) CCD 841 CoN cells were treated with either DMSO or 15-keto PGE$_2$ (20 and 40 μM) for 24 h. The whole lysates were subjected to immunoblot analysis for the measurement of HO-1. GAPDH was used as an equal loading control for normalization. (B) Immunofluorescent staining was done for assessing HO-1 expression in the CCD 841 CoN cells treated with 15-keto PGE$_2$ (40 μM) or vehicle for 12 h. The cells were fixed with 2% buffered formaldehyde solution and blocked with 3% BSA. The cells were immunoblotted with anti-HO-1 primary antibody and a fluorophore-conjugated secondary antibody. DAPI was applied for staining nucleus before detection of HO-1 by fluorescence microscopy. (C, D) CCD 841 CoN cells were treated with 15-keto PGE$_2$ (40 μM) for the indicated periods. The mRNA level of antioxidant proteins was determined by RT-PCR (C). The lysates were subjected to immunoblot analysis for measuring the protein levels of HO-1, TXN, and NQO-1 (D). Bar graphs show means ± S.E. from at least 3 replicated experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Following fixation of cells by incubation in PBS with 2% formaldehyde at room temperature for 10–15 min, the fixed cells were permeabilized for 5 min at room temperature using 0.2% Triton X-100 in PBS. The cells were blocked with 3% bovine serum albumin (BSA) in PBS containing 0.1% Triton X-100 for 1 h at room temperature 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 Fluorophore-conjugated secondary antibodies (Alexa fluor® 488) for another 1 h at room temperature. Samples were washed with 0.05% PBST containing 3% BSA and then examined under an LSM 700 confocal microscope (ZEISS; Oberkochen, Germany) integrated by ZEN imaging software.

2.7. Transfection

The small interfering RNA (siRNA) of Nrf2 and its negative control siRNA were purchased from Genolution Pharmaceuticals (Seoul, Korea). The sequence of human specific Nrf2-siRNA used is as follows: sense 5′-GGGAGGAGCUAUUAUCCAUUU-3′; antisense 5′-AUGGAUAAUAGCUCCUCCCUU-3′. Human CCD 841 CoN cells (4×10^5/6-well) were transfected with 20 nM of oligonucleotides using Lipofectamine RNAiMAX according to manufacturer's instructions (Thermo Fisher Scientific; Waltham, MA, USA).

2.8. ARE luciferase reporter gene assay

CCD 841 CoN cells were plated at a confluence of 50% density in 6-well plate and grown in MEM media supplemented with 5% heat-inactivated horse serum at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were co-transfected with 1 μg of the luciferase reporter gene fusion construct (pTi-luciferase), wild-type ARE, and 0.5 μg of pCMV-β-galactosidase control vector with WelFect-M™ Gold transfection reagent according to the manufacturer's instructions. After 24-h transfection, the cells were treated with 15-keto PGE_2 for additional 12 h and the cell lysis was carried out with the 1x reporter lysis buffer (Promega; Madison, WI, USA). After mixing the cell extract with a luciferase substrate (Promega, Madison, WI, USA), the luciferase activity was measured by the luminometer. The β-galactosidase assay was done according to the supplier's instructions (Promega β-galactosidase Enzyme Assay System) for normalizing the luciferase activity.
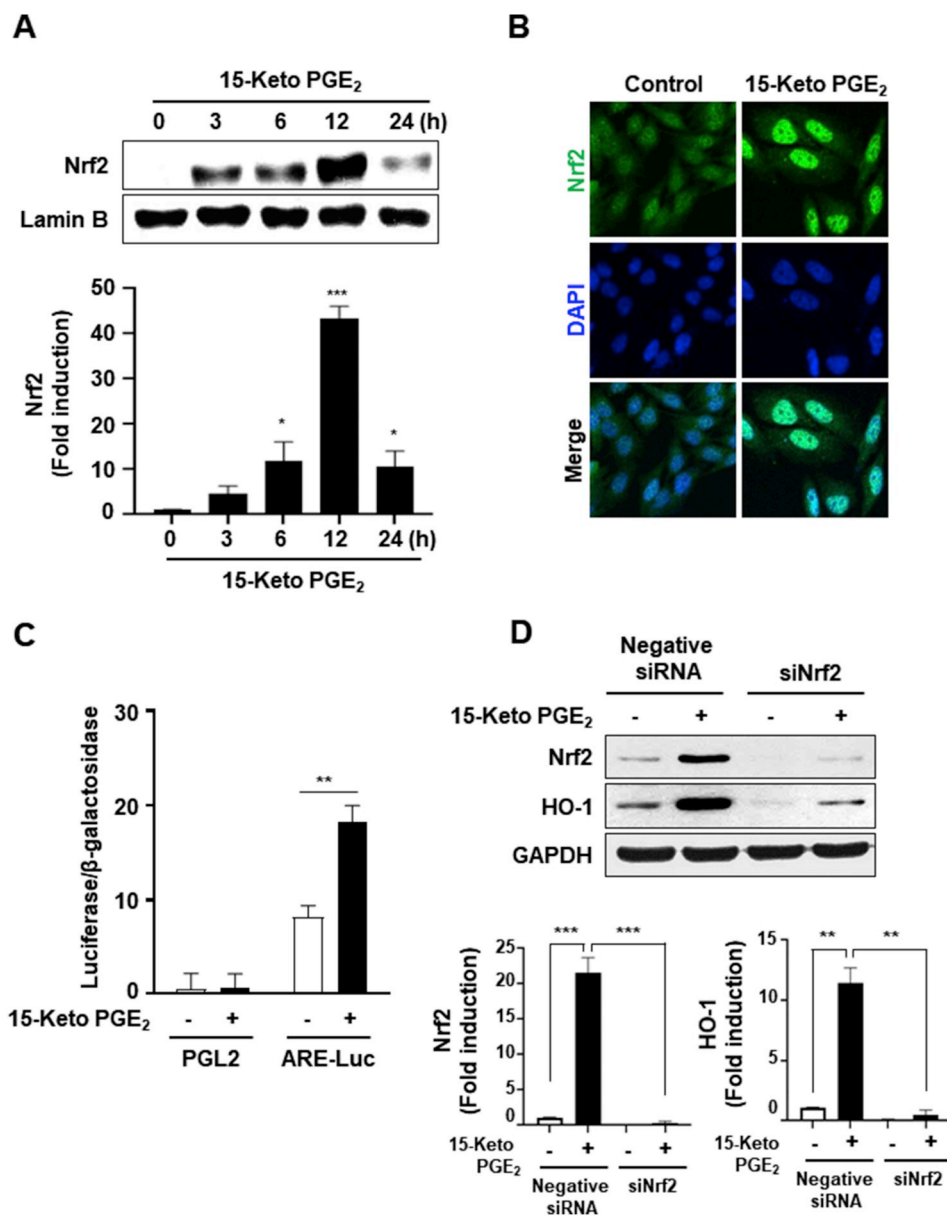

Fig. 2. 15-Keto PGE_2-induced HO-1 expression mediated by Nrf2. (A) CCD 841 CoN cells were treated with 15-keto PGE_2 (40 μM) for the indicated time periods. The nuclear fractions were subjected to immunoblot analysis for the measurement of Nrf2. Lamin B1 was used as a nuclear protein marker. Western blot analysis was conducted at least 3 times and the blot is the representative one. (B) The effect of 15-keto PGE_2 (40 μM) on the nuclear localization of Nrf2 was determined by immunocytochemical analysis. (C) CCD 841 CoN cells were co-transfected with pCMV-β-galactosidase and either the luciferase reporter gene fusion construct (pGL2) or ARE luciferase reporter gene for 36 h, followed by treatment with DMSO or 15-keto PGE_2 (40 μM) for additional 12 h. The transcriptional activity of Nrf2 was assessed based on the ARE-driven luciferase activity. (D) CCD 841 CoN cells were transfected with siRNA negative control or siNrf2 for 12 h and exposed to 15-keto PGE_2 (40 μM) for another 12 h. The lysates from transfected cells were subjected to Western blot analysis. All graphs show the mean ± S.E. (n = 3). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

2.9. Measurement of intracellular reactive oxygen species (ROS)

Accumulation of ROS in CCD 841 CoN cells treated with 15-keto PGE_2 was monitored using the fluorescence-generating probe, DCF-DA. The cells (1×10^5/mL) were then washed once with HBSS and treated with 10 mM DCF-DA to assess ROS-mediated oxidation of DCF-DA to the fluorescent compound DCF. After 30-min incubation at 37 °C, the cells were examined under a confocal fluorescence microscope set at 488 nm for excitation and 530 nm for emission.

2.10. Statistical analysis

All data are analyzed by means ± S.E. and are based on experiments performed at least in triplicate. Statistical significance was calculated with the Student's *t*-test and GraphPad Prism 8 (San Diego, CA, USA) software.

3. Results

3.1. 15-Keto PGE_2 upregulates expression of HO-1 in CCD 841 CoN cells

We initially selected non-cytotoxic concentrations of 15-keto-PGE_2 in human normal colon epithelial CCD 841 CoN cells by the MTT assay.
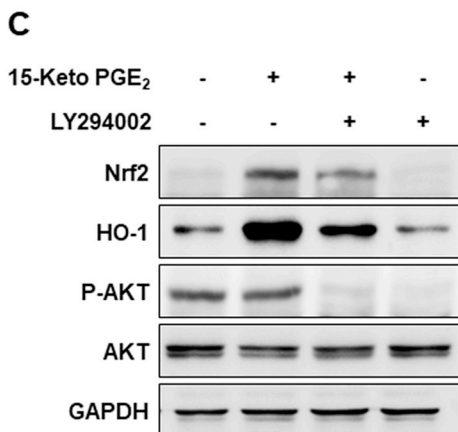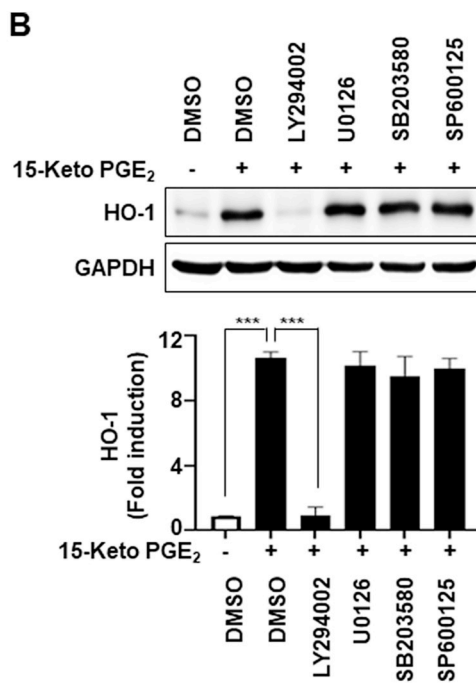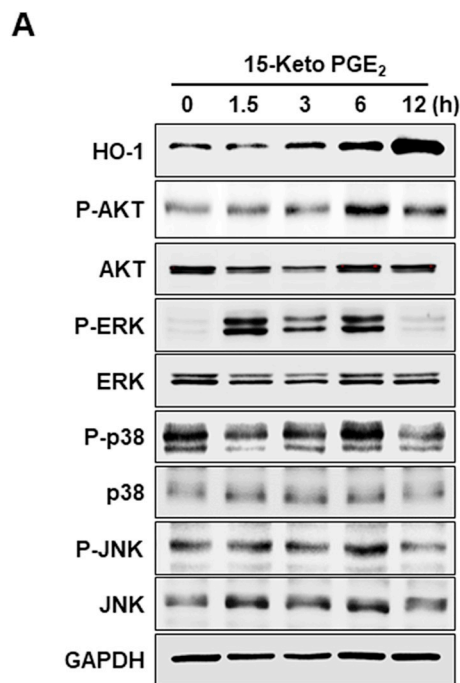
The concentrations (20 μM and 40 μM) used in this experiment did not show any cytotoxic effect up to 24 h. We observed that 15-keto PGE_2 (40 μM) induced the expression of HO-1 as determined by Western blot analysis (Fig. 1A). The induction of HO-1 by 15-keto-PGE_2 was also verified by immunocytochemical analysis (Fig. 1B). To determine whether 15-keto PGE_2 could induce expression of other antioxidant enzymes such as TXN and NQO1, CCD 841 CoN cells were treated with this lipid mediator for different periods (3 h, 6 h, 12 h, and 24 h). While both mRNA and protein expression of HO-1 was upregulated at 6 h and sustained up to 12 h upon 15-keto PGE_2 treatment, the expression of TXN and NQO1 was not induced (Fig. 1C and D).

3.2. 15-Keto PGE_2 stimulates the nuclear translocation and transcriptional activity of Nrf2

As Nrf2 is the primary transcription factor for regulating the expression of HO-1, we investigated whether 15-keto PGE_2 could affect the activation of Nrf2. 15-Keto PGE_2 induced the nuclear translocation of Nrf2 which was evident at 3 h after the treatment and gradually increased up to 12 h (Fig. 2A). This was also observed by immunocytochemical analysis (Fig. 2B). In addition, we assessed the transcriptional activity of Nrf2 by transfecting CCD 841 CoN cells with an ARE driven-luciferase vector. Treatment of CCD 841 CoN cells with

Fig. 3. The role of AKT in 15-keto PGE_2-induced HO-1 expression. (A) Effects of 15-keto PGE_2 on the activity of protein kinases including AKT, ERK, p38, and JNK. The cells were treated with 15-keto PGE_2 (40 μM) for the indicated time. (B) Effects of kinase inhibitors on 15-keto PGE_2-induced HO-1 expression. Cells were pre-incubated for 1 h with LY294002 (10 μM), U0126 (5 μM), SB203580 (10 μM), or SP600125 (10 μM), followed by 15-keto PGE_2 treatment for 12 h. The lysates were subjected to immunoblot analysis for the measurement of HO-1. (C) Effects of AKT inhibition on 15-keto PGE_2-induced expression of Nrf2 and HO-1. Cells were treated with 15-keto PGE_2 (40 μM) for 12 h in the absence or presence of LY294002 (10 μM). The lysates were subjected to immunoblot analysis for the measurement of Nrf2, HO-1, AKT, and P-AKT. Data are expressed as the mean ± S.E. (n = 3). ***$p < 0.001$.

15-keto PGE$_2$ significantly enhanced the luciferase intensity in cells transfected with ARE driven-luciferase vector (Fig. 2C). We further determined whether 15-keto PGE$_2$-induced Nrf2 activation is responsible for the induction of HO-1 expression. For this purpose, cells were transfected with either negative siRNA or Nrf2 siRNA. We observed that the silencing of Nrf2 abolished the 15-keto PGE$_2$-mediated upregulation of HO-1 (Fig. 2D). This finding suggests that Nrf2 plays an important role in the induction of HO-1 expression by 15-keto PGE$_2$.

3.3. 15-Keto PGE$_2$-induced Nrf2-HO-1 upregulation is mediated through AKT activation

Dissociation of the Nrf2-Keap1 complex is stimulated through the phosphorylation of Nrf2 by distinct upstream kinases. To determine the upstream signaling events that lead to activation of Nrf2 and consequent HO-1 expression, we assessed activation of several kinases including AKT and mitogen-activated protein kinases such as ERK, JNK, and p38. We found that 15-keto PGE$_2$ induced the phosphorylation of AKT and ERK1/2 (Fig. 3A). The pharmacological inhibitor of AKT (LY294002) attenuated the 15-keto PGE$_2$-induced expression of HO-1, while U0126 (MEK inhibitor), SB203580 (p38 inhibitor) and SP600125 (JNK inhibitor) exerted no inhibitory effect (Fig. 3B). Further, blockade of AKT activity with LY294002 attenuated accumulation of Nrf2 and expression of HO-1 (Fig. 3C), suggesting that activation of the Nrf2/HO-1 axis by 15-keto PGE$_2$ is mediated by AKT .

3.4. 15-Keto PGE$_2$ stimulates the generation of ROS

Although both excess and insufficient ROS impair normal physiologic functions, a moderate generation of ROS is necessary for the
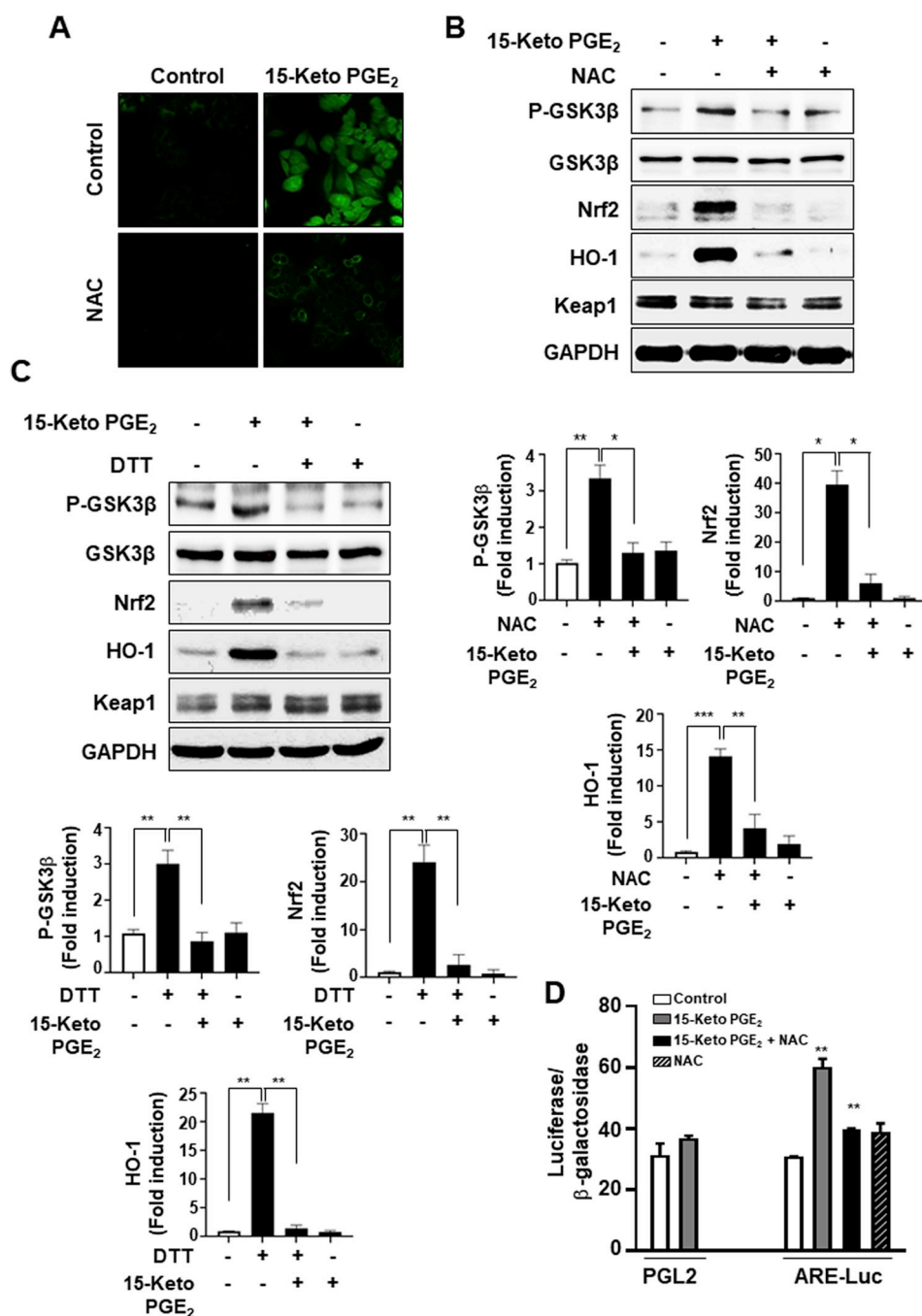

Fig. 4. 15-Keto PGE$_2$-induced generation of ROS. (A) Generation of ROS in CCD 841 CoN cells treated with 15-keto PGE$_2$ was monitored using the fluorescence-generating probe DCF-DA. CCD 841 CoN cells were incubated with 15-keto PGE$_2$ (40 μM) in the absence or presence of NAC (5 mM) for 12 h. (B, C) CCD 841 CoN cells were incubated with 15-keto PGE$_2$ (40 μM) in the absence or presence of NAC (5 mM) (B) or DTT (0.5 mM) (C) for 12 h. The expression levels of P-GSK3β, GSK-3β, Nrf2, Keap1, and HO-1 were measured by the Western blot analysis. (D) CCD 841 CoN cells were co-transfected with pCMV-β-galactosidase and either the luciferase reporter gene fusion construct (pGL2) or ARE luciferase reporter gene for 36 h, followed by treatment with DMSO, 15-keto PGE$_2$, 15-keto PGE$_2$ plus NAC, or NAC alone for 12 h. The transcriptional activity of Nrf2 was measured by the ARE luciferase reporter assay as described in Methods. Bar graphs show the mean ± S.E. (n = 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

maintenance of cellular redox balance and homeostasis [24]. Nrf2 is adaptively activated upon intracellular oxidative stress. When we examined the level of ROS in CCD 841 CoN cells treated with 15-keto PGE$_2$ using DCF-DA staining, there was a generation of intracellular ROS, which was abolished by a general antioxidant NAC (Fig. 4A). NAC treatment also attenuated the accumulation of Nrf2 and expression of HO-1 (Fig. 4B). GSK3β, a downstream kinase of AKT, has been known to stimulate Nrf2 degradation, independently of Keap1 signaling [25]. GSK3β undergoes inactivation through phosphorylation at Ser9. 15-Keto PGE$_2$ induced phosphorylation of GSK3β^{Ser9}, which was abolished by NAC (Fig. 4B). Similar results were achieved with another reducing agent DTT (Fig. 4C). Moreover, 15-keto PGE$_2$-induced transcriptional activity of Nrf2 was also attenuated by NAC (Fig. 4D). These findings suggest that ROS production induced by 15-keto PGE$_2$ contributes to the activation of Nrf2 and the expression of HO-1 through activation of the GSK3β pathway.

3.5. The α,β-unsaturated carbonyl group of 15-keto PGE$_2$ is critical for Nrf2 activation and subsequent HO-1 expression

15-Keto-PGE$_2$ has an α,β-unsaturated carbonyl moiety which is capable of interacting with nucleophilic cellular proteins [26]. The biological function of many electrophilic compounds has been attributable to their α,β-unsaturated carbonyl groups [27,28]. 15-Keto PGE$_2$ is converted by prostaglandin reductase 2 (PTGR2) to 13,14-di-hydro-15-keto PGE$_2$ lacking an electrophilic α,β-unsaturated carbonyl group [29] (Fig. 5A). As shown in Fig. 5B, 13,14-dihydro-15-keto PGE$_2$ failed to generate ROS, while 15-keto-PGE$_2$ did (Fig. 5B). Further, the accumulation of Nrf2 and expression of HO-1 were less pronounced in the cells treated with 13,14-dihydro-15-keto PGE$_2$ compared to those treated with 15-keto PGE$_2$ (Fig. 5C). These findings indicate that the α,β-unsaturated carbonyl group of 15-keto PGE$_2$ is critical for Nrf2 activation and subsequent HO-1 expression.

3.6. 15-Keto PGE$_2$-induced HO-1 expression may contribute to protecting cells against oxidative cell death and inflammation

Induction of HO-1 has been known to protect the cells against oxidative or inflammatory stress [30]. Treatment of CCD 841 CoN cells for 6 h with hydrogen peroxide (H$_2$O$_2$) induced apoptosis as evidenced by the cleavage of poly(ADP-ribose) polymerase 1 (PARP-1) and down-regulation of Bcl-2 phosphorylation (Fig. 6A). The pre-treatment with 15-keto PGE$_2$ for 6 h attenuated both pro-apoptotic events in H$_2$O$_2$-treated cells (Fig. 6A). The transcription factor STAT3 plays a pivotal role in inflammation and inflammation-associated carcinogenesis [31]. Phosphorylation at the Tyr^{705} is essential for the activation of STAT3. 15-Keto
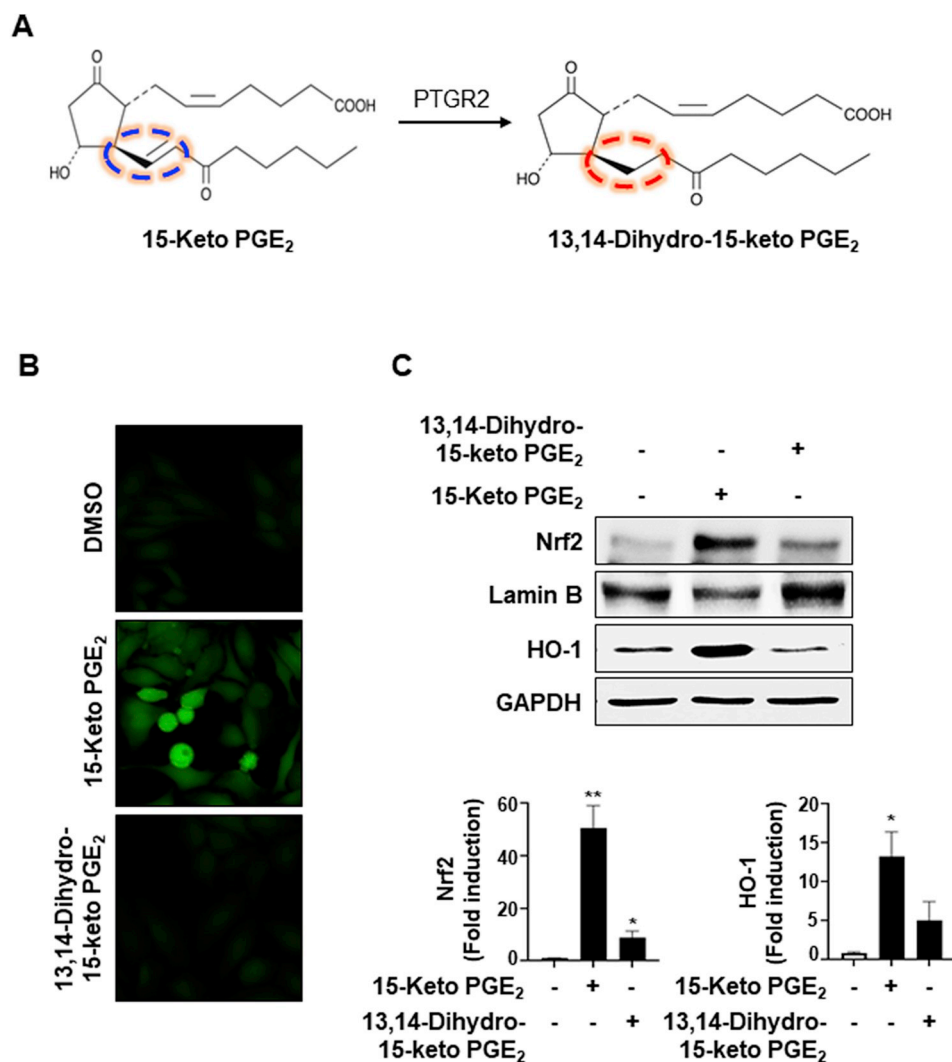

Fig. 5. The importance of α,β-unsaturated carbonyl group for 15-keto PGE$_2$-induced Nrf2 activation and subsequent HO-1 expression. (A) 15-Keto PGE$_2$ possesses the α,β-unsaturated carbonyl moiety which has a chemically reactive carbon center, whereas 13,14-dihydro-15-keto PGE$_2$ lacks this electrophilic moiety. (B) The comparison of ROS generation in the cells treated with DMSO, 15-keto-PGE$_2$ (40 μM) or 13,14-dihydro-15-keto PGE$_2$ (40 μM). (C) The effect of 15-keto PGE$_2$ (40 μM) and 13,14-dihydro-15-keto PGE$_2$ (40 μM) on nuclear localization of Nrf2 and expression of HO-1. The expression of Nrf2 and HO-1 was determined by Western blot analysis. GAPDH and Lamin B were used as an equal loading control and a nuclear protein marker, respectively. *$p < 0.05$, **$p < 0.01$.
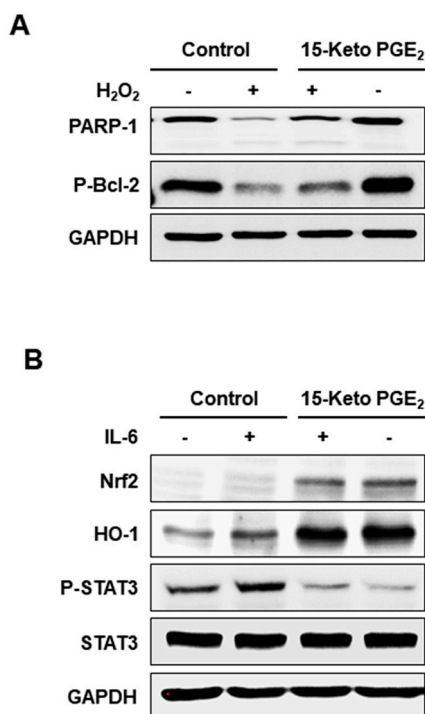
A

B

Fig. 6. Protective effects of 15-keto PGE$_2$ on oxidative stress-induced apoptosis and IL-6-induced activation of STAT3 signaling. (A) H$_2$O$_2$ (300 μM) was treated to CCD 841 CoN cells in the absence or presence of 15-keto PGE$_2$ (40 μM) for 6 h. Cleavage of PARP-1 and phosphorylation of Bcl-2 were evaluated by Western blot analysis. (B) The pro-inflammatory cytokine IL-6 was treated to CCD 841 CoN cells for 3 h in the absence or presence of 15-keto PGE$_2$ (40 μM) for 12 h. Nrf2, HO-1, phosphorylated STAT3, and STAT3 were detected by Western blot analysis.

PGE$_2$ inhibited the STAT3 phosphorylation induced by the pro-inflammatory cytokine IL-6 in CCD 841 CoN cells (Fig. 6B). These findings suggest that 15-keto PGE$_2$-induced expression of HO-1 may contribute to protecting cells against oxidative cell death and inflammation.
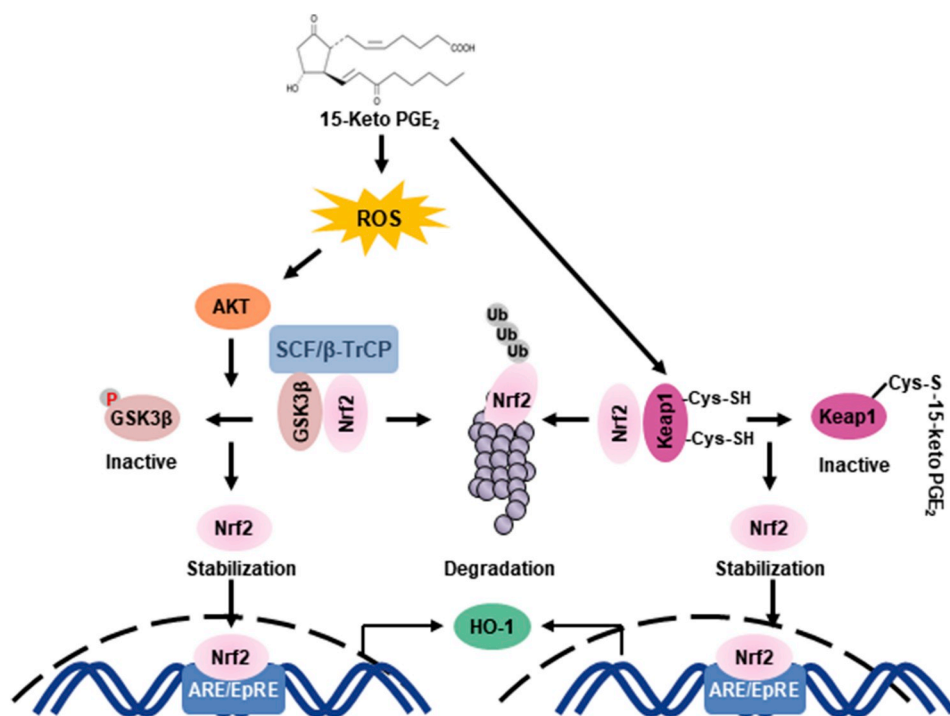
4. Discussion

Inflammatory disorders including inflammation-associated cancer are largely driven by the generation of a proinflammatory PGE$_2$. This lipid mediator governs inflammatory responses by eliciting vasodilatation and enhancing vascular permeability, thereby mediating the influx of leukocytes [32,33]. In many solid tumors, PGE$_2$ is often over-produced [34-36] and is tightly linked to chemoresistance [37]. Although suppression of PGE$_2$ generation by COX-2 selective inhibitors could be a therapeutic option in the management of inflammation-associated cancer, this may also inhibit the production of certain downstream lipid mediators which may jeopardize the resolution of inflammation [38,39].

Recently, 15-keto PGE$_2$ is considered as a novel lipid mediator possessing anti-inflammatory properties. We observed that 15-keto PGE$_2$ induces HO-1 expression through Nrf2 activation, which contributes to its anti-inflammatory and anti-oxidative effects. Consistently, Chen et al. have also reported that 15-keto-PGE$_2$ induces HO-1 via Nrf2 activation in macrophages [15]. Nrf2-mediated HO-1 induction contribute to adaptive survival response [40]. Upon activation of Nrf2, upregulation of HO-1 expression not only mediates an antioxidant reaction but also facilitates anti-inflammatory, antiapoptotic and antiproliferative events [41]. In our present study, 15-keto PGE$_2$ suppressed the H$_2$O$_2$-induced apoptosis and phosphorylation of STAT3 induced by IL-6. Therefore, anti-inflammatory and cytoprotective effects of 15-keto PGE$_2$ may be largely mediated through activation of Nrf2-HO-1 signaling.

Nrf2 plays a pivotal role not only in the cellular defense against oxidative stress but also in protection against inflammatory disorders [19]. Mice deficient in Nrf2 developed more severe colitis upon treatment with DSS than wild type mice [42]. Likewise, the levels of pro-inflammatory mediators, such as COX-2, were remarkably increased in the colonic tissues of Nrf2$^{-/-}$ mice as compared to their wild-type counterparts [42]. In addition, Nrf2 is a critical suppressor of colon cancer progression as well as inflammation. Nrf2 knockout mice were more susceptible to the formation of colorectal cancer than wild-type mice treated with the carcinogen azoxymethane plus DSS [43]. Notably, Nrf2 activation exhibited a preventive effect on the initiation of

Fig. 7. A proposed scheme for activation of Nrf2 by 15-keto PGE$_2$. 15-Keto PGE$_2$ may stabilize and stimulate accumulation of Nrf2 by blocking its degradation. In one way, it induces the generation of intracellular ROS and subsequent activation of AKT. AKT triggers phosphorylation of GSK3β and results in deactivation of this kinase, thereby attenuating SCF/β-TrCP-mediated Nrf2 degradation. In another way, 15-keto PGE$_2$ reacts with the cysteine residue of Keap1 and blocks the Keap1-dependent degradation of Nrf2. The stabilized Nrf2 translocates into the nucleus and regulates the expression of HO-1.

pulmonary cancer [44], although it can be hijacked by cancer cells, leading to stimulation of cancer progression [44,45]. It appears that Nrf2 inhibits the initiation of carcinogenesis, but may function as an oncogenic protein once cells are transformed to (pre)cancerous ones [46,47].

15-Keto PGE$_2$ has an α,β-unsaturated carbonyl moiety and is metabolized to non-electrophilic 13,14-dihydro-15-keto PGE$_2$ by PTGR [29]. We have observed that the α,β-unsaturated carbonyl moiety of 15-keto PGE$_2$ plays an important role in activating Nrf2 as 13,14-dihydro-15-keto PGE$_2$ was inactive in this regard. 15-Keto PGE$_2$ covalently reacts with free cysteine residues of proteins involved in the regulation of the intracellular signaling pathways. Our recent study reveals a reaction of the α,β unsaturated carbonyl group of 15-keto PGE$_2$ with the Cys259 residue present in STAT3 [18], which accounts for its anticarcinogenic response. The canonical activation of Nrf2 is carried out by oxidation of cysteine residues present in the inhibitory protein Keap1 [48] including Cys151, 273 and 288 [49]. The cysteine modification of Keap1 by electrophiles either changes its conformation or deactivates the Keap1-ubiquitin E3 ligase complex, resulting in a dissociation of Nrf2 from Keap1 or blockade of ubiquitination-related degradation of Nrf2, respectively. It has recently been reported that 15-keto PGE$_2$ covalently binds to the Cys288 residue of Keap1, which may account for its inhibition of pro-inflammatory cytokine production and induction of antioxidant gene expression in murine macrophages [15]. Consistent with the above study, our data on the attenuation of 15-keto PGE$_2$-induced Nrf2 accumulation and HO-1 expression by NAC and DTT suggest the Keap1 cysteine thiol modification as an underlying mechanism (Fig. 7). Another electrophilic lipid mediator, 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$ (15d-PGJ$_2$), modifies cysteine residues of target proteins such as phosphatase and tensin homolog (PTEN) and carries out distinct biological functions [50]. 15d-PGJ$_2$ induces the expression of HO-1 via activation of Nrf2 signaling in macrophages, which is associated with efferocytosis, a key event in the resolution of inflammation [51].

Activation of Nrf2 is regulated by AKT/GSK-3β signaling [52,53]. GSK-3β, downstream of AKT signaling, has been known to phosphorylate a group of Ser residues in the Neh6 domain of mouse Nrf2 and promotes its degradation by E3 ubiquitin ligase SCF/β-TrCP [25]. AKT phosphorylates GSK-3β, thereby inactivating the latter kinase, which contributes to Nrf2 activation [53]. Based on this finding, phosphorylation of AKT and GSK3β by 15-keto PGE$_2$ likely suppresses Nrf2 degradation mediated by SCF/β-TrCP even without affecting Keap1 (Fig. 7). It has been reported that hydrogen peroxide generated by 15d-PGJ$_2$ oxidizes and inactivates PTEN, leading to activation of AKT [50]. In line with this speculation, one plausible mechanism by which 15-keto PGE$_2$ activates AKT signaling may involve oxidative inactivation of the PTEN, a negative regulator of PI3K-AKT, through the generation of ROS.

It has been reported that the silencing of PTGR2 or treatment with 15-keto-PGE$_2$ increases ROS production, which leads to pancreatic cancer cell death [17]. In this study, 15-keto PGE$_2$ reduces the level of GSH, a first-line of cellular antioxidant defense, and expression of solute carrier family 7 member 11 and cystathionine γ-lyase which are involved in the production of cysteine and subunit of the cystine/glutamate antiporter [17]. Based on these findings, 15-keto PGE$_2$-induced generation ROS may occur as a consequence of a low level of GSH in colon normal epithelial cells.

In conclusion, 15-keto PGE$_2$ stimulates the Nrf2-HO-1 axis through the generation of ROS in normal colon epithelial cells, which contributes to the potential role of this lipid mediator in preventing cancer development associated with oxidative stress and inflammatory tissue damage.

Declaration of competing interest

The authors declare no competing financial interests.

References

[1] C. Luceri, E. Bigagli, S. Agostiniani, F. Giudici, D. Zambonin, S. Scaringi, F. Ficari, M. Lodovici, C. Malentacchi, Analysis of oxidative stress-related markers in Crohn's disease patients at surgery and correlations with clinical findings, Antioxidants (Basel) 8 (2019) E378.

[2] Singer II, D.W. Kawka, S. Schloemann, T. Tessner, T. Riehl, W.F. Stenson, Cyclooxygenase 2 is induced in colonic epithelial cells in inflammatory bowel disease, Gastroenterology 115 (1998) 297–306.

[3] R.A. Gupta, R.N. Dubois, Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2, Nat. Rev. Cancer 1 (2001) 11–21.

[4] J.Y. Park, M.H. Pillinger, S.B. Abramson, Prostaglandin E$_2$ synthesis and secretion: the role of PGE$_2$ synthases, Clin. Immunol. 119 (2006) 229–240.

[5] S. Pugh, G.A. Thomas, Patients with adenomatous polyps and carcinomas have increased colonic mucosal prostaglandin E$_2$, Gut 35 (1994) 675–678.

[6] L. Dai, D.W. King, D.S. Perera, D.Z. Lubowski, E. Burcher, L. Liu, Inverse expression of prostaglandin E$_2$-related enzymes highlights differences between diverticulitis and inflammatory bowel disease, Dig. Dis. Sci. 60 (2015) 1236–1246.

[7] M.G. Backlund, J.R. Mann, V.R. Holla, F.G. Buchanan, H.H. Tai, E.S. Musiek, G.L. Milne, S. Katkuri, R.N. DuBois, 15-Hydroxyprostaglandin dehydrogenase is down-regulated in colorectal cancer, J. Biol. Chem. 280 (2005) 3217–3223.

[8] A. Thiel, A. Ganesan, J. Mrena, S. Junnila, A. Nykanen, A. Hemmes, H.H. Tai, O. Monni, A. Kokkola, C. Haglund, T.V. Petrova, A. Ristimaki, 15-Hydroxyprostaglandin dehydrogenase is down-regulated in gastric cancer, Clin. Cancer Res. 15 (2009) 4572–4580.

[9] J.E. Yang, E. Park, H.J. Lee, H.J. Kang, K.M. Kim, E. Yu, D. Lee, J.H. Shim, Y.S. Lim, H.C. Lee, Y.H. Chung, Y.S. Lee, Role of 15-hydroxyprostaglandin dehydrogenase down-regulation on the prognosis of hepatocellular carcinoma, Clin. Mol. Hepatol. 20 (2014) 28–37.

[10] K. Arima, Y. Komohara, L. Bu, M. Tsukamoto, R. Itoyama, K. Miyake, T. Uchihara, Y. Ogata, S. Nakagawa, H. Okabe, K. Imai, D. Hashimoto, A. Chikamoto, Y.I. Yamashita, H. Baba, T. Ishimoto, Downregulation of 15-hydroxyprostaglandin dehydrogenase by interleukin-1β from activated macrophages leads to poor prognosis in pancreatic cancer, Cancer Sci. 109 (2018) 462–470.

[11] I. Wolf, J. O'Kelly, T. Rubinek, M. Tong, A. Nguyen, B.T. Lin, H.H. Tai, B.Y. Karlan, H.P. Koeffler, 15-Hydroxyprostaglandin dehydrogenase is a tumor suppressor of human breast cancer, Cancer Res. 66 (2006) 7818–7823.

[12] L.M. Mehdawi, S.R. Satapathy, A. Gustafsson, K. Lundholm, M. Alvarado-Kristensson, A. Sjolander, A potential anti-tumor effect of leukotriene C4 through the induction of 15-hydroxyprostaglandin dehydrogenase expression in colon cancer cells, Oncotarget 8 (2017) 35033–35047.

[13] S.H. Choi, B.G. Kim, J. Robinson, S. Fink, M. Yan, M.B. Sporn, S.D. Markowitz, J.J. Letterio, Synthetic triterpenoid induces 15-PGDH expression and suppresses inflammation-driven colon carcinogenesis, J. Clin. Invest. 124 (2014) 2472–2482.

[14] H.K. Na, J.M. Park, H.G. Lee, H.N. Lee, S.J. Myung, Y.J. Surh, 15-Hydroxyprostaglandin dehydrogenase as a novel molecular target for cancer chemoprevention and therapy, Biochem. Pharmacol. 82 (2011) 1352–1360.

[15] I.J. Chen, S.W. Hee, C.H. Liao, S.Y. Lin, L. Su, C.T. Shun, L.M. Chuang, Targeting the 15-keto-PGE$_2$-PTGR2 axis modulates systemic inflammation and survival in experimental sepsis, Free Radic. Biol. Med. 115 (2018) 113–126.

[16] D. Lu, C. Han, T. Wu, 15-PGDH inhibits hepatocellular carcinoma growth through 15-keto-PGE$_2$/PPARγ-mediated activation of p21$^{WAF1/Cip1}$, Oncogene 33 (2014) 1101–1112.

[17] E.Y. Chang, Y.C. Chang, C.T. Shun, Y.W. Tien, S.H. Tsai, S.W. Hee, I.J. Chen, L.M. Chuang, Inhibition of prostaglandin reductase 2, a putative oncogene over-expressed in human pancreatic adenocarcinoma, induces oxidative stress-mediated cell death involving *xCT* and *CTH* gene expressions through 15-keto-PGE$_2$, PLoS One 11 (2016) e0147390.

[18] E.J. Lee, Y.I. Hahn, S.J. Kim, H.J. Yoon, B. Hane, S.-J.K. Young-Il Hahn, Hyo-Jin Yoon, Bitnara Hane, Kyeojin Kim, Seungbeom Lee, Kwang Pyo Kim, Ger Suh Young, Hye-Kyung Na, Young-Joon Surh, 15-Keto prostaglandin E$_2$ suppresses STAT3 signaling and inhibits breast cancer cell growth and progression, Redox Biol. 23 (2019) 101175 .

[19] S.M. Ahmed, L. Luo, A. Namani, X.J. Wang, X. Tang, Nrf2 signaling pathway: pivotal roles in inflammation, Biochim. Biophys. Acta Mol. Basis Dis. 1863 (2017) 585–597.

[20] Y.J. Surh, J.K. Kundu, H.K. Na, Nrf2 as a master redox switch in turning on the cellular signaling involved in the induction of cytoprotective genes by some chemopreventive phytochemicals, Planta Med. 74 (2008) 1526–1539.

[21] D.D. Zhang, S.C. Lo, Z. Sun, G.M. Habib, M.W. Lieberman, M. Hannink, Ubiquitination of Keap1, a BTB-Kelch substrate adaptor protein for Cul3, targets Keap1 for degradation by a proteasome-independent pathway, J. Biol. Chem. 280 (2005) 30091–30099.

[22] A.T. Dinkova-Kostova, W.D. Holtzclaw, R.N. Cole, K. Itoh, N. Wakabayashi, Y. Katoh, M. Yamamoto, P. Talalay, Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 11908–11913.

[23] N. Wakabayashi, A.T. Dinkova-Kostova, W.D. Holtzclaw, M.I. Kang, A. Kobayashi, M. Yamamoto, T.W. Kensler, P. Talalay, Protection against electrophile and oxidant

stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 2040–2045.

[24] W. Droge, Free radicals in the physiological control of cell function, Physiol. Rev. 82 (2002) 47–95.

[25] P. Rada, A.I. Rojo, S. Chowdhry, M. McMahon, J.D. Hayes, A. Cuadrado, SCF/β-TrCP promotes glycogen synthase kinase 3-dependent degradation of the Nrf2 transcription factor in a Keap1-independent manner, Mol. Cell. Biol. 31 (2011) 1121–1133.

[26] T. Shiraki, N. Kamiya, S. Shiki, T.S. Kodama, A. Kakizuka, H. Jingami, α,β-Unsaturated ketone is a core moiety of natural ligands for covalent binding to peroxisome proliferator-activated receptor γ, J. Biol. Chem. 280 (2005) 14145–14153.

[27] S. Amslinger, The tunable functionality of α,β-unsaturated carbonyl compounds enables their differential application in biological systems, ChemMedChem 5 (2010) 351–356.

[28] T. Rodrigues, D. Reker, P. Schneider, G. Schneider, Counting on natural products for drug design, Nat. Chem. 8 (2016) 531–541.

[29] Y.H. Wu, T.P. Ko, R.T. Guo, S.M. Hu, L.M. Chuang, A.H. Wang, Structural basis for catalytic and inhibitory mechanisms of human prostaglandin reductase PTGR2, Structure 16 (2008) 1714–1723.

[30] L.A. Applegate, P. Luscher, R.M. Tyrrell, Induction of heme oxygenase: a general response to oxidant stress in cultured mammalian cells, Cancer Res. 51 (1991) 974–978.

[31] J. Huynh, A. Chand, D. Gough, M. Ernst, Therapeutically exploiting STAT3 activity in cancer - using tissue repair as a road map, Nat. Rev. Cancer 19 (2019) 82–96.

[32] J.L. Wallace, Prostaglandin biology in inflammatory bowel disease, Gastroenterol. Clin. North Am. 30 (2001) 971–980.

[33] S. Hara, D. Kamei, Y. Sasaki, A. Tanemoto, Y. Nakatani, M. Murakami, Prostaglandin E synthases: understanding their pathophysiological roles through mouse genetic models, Biochimie 92 (2010) 651–659.

[34] B. Rigas, I.S. Goldman, L. Levine, Altered eicosanoid levels in human colon cancer, J. Lab. Clin. Med. 122 (1993) 518–523.

[35] K. Uefuji, T. Ichikura, H. Mochizuki, Cyclooxygenase-2 expression is related to prostaglandin biosynthesis and angiogenesis in human gastric cancer, Clin. Cancer Res. 6 (2000) 135–138.

[36] P.H. Rolland, P.M. Martin, J. Jacquemier, A.M. Rolland, M. Toga, Prostaglandin in human breast cancer: evidence suggesting that an elevated prostaglandin production is a marker of high metastatic potential for neoplastic cells, J. Natl. Cancer Inst. 64 (1980) 1061–1070.

[37] A.V. Kurtova, J. Xiao, Q. Mo, S. Pazhanisamy, R. Krasnow, S.P. Lerner, F. Chen, T.T. Roh, E. Lay, P.L. Ho, K.S. Chan, Blocking PGE$_2$-induced tumour repopulation abrogates bladder cancer chemoresistance, Nature 517 (2015) 209–213.

[38] K. Fukunaga, P. Kohli, C. Bonnans, L.E. Fredenburgh, B.D. Levy, Cyclooxygenase 2 plays a pivotal role in the resolution of acute lung injury, J. Immunol. 174 (2005)

5033–5039.

[39] J.U. Scher, M.H. Pillinger, The anti-inflammatory effects of prostaglandins, J. Investig. Med. 57 (2009) 703–708.

[40] S.H. Park, J.H. Jang, C.Y. Chen, H.K. Na, Y.J. Surh, A formulated red ginseng extract rescues PC12 cells from PCB-induced oxidative cell death through Nrf2-mediated upregulation of heme oxygenase-1 and glutamate cysteine ligase, Toxicology 278 (2010) 131–139.

[41] J.A. Araujo, M. Zhang, F. Yin, Heme oxygenase-1, oxidation, inflammation, and atherosclerosis, Front. Pharmacol. 3 (2012) 119.

[42] T.O. Khor, M.T. Huang, K.H. Kwon, J.Y. Chan, B.S. Reddy, A.N. Kong, Nrf2-deficient mice have an increased susceptibility to dextran sulfate sodium-induced colitis, Cancer Res. 66 (2006) 11580–11584.

[43] T.O. Khor, M.T. Huang, A. Prawan, Y. Liu, X. Hao, S. Yu, W.K. Cheung, J.Y. Chan, B.S. Reddy, C.S. Yang, A.N. Kong, Increased susceptibility of Nrf2 knockout mice to colitis-associated colorectal cancer, Cancer Prev. Res. (Phila) 1 (2008) 187–191.

[44] S. Tao, M. Rojo de la Vega, E. Chapman, A. Ooi, D.D. Zhang, The effects of NRF2 modulation on the initiation and progression of chemically and genetically induced lung cancer, Mol. Carcinog. 57 (2018) 182–192.

[45] M. Rojo de la Vega, E. Chapman, D.D. Zhang, NRF2 and the hallmarks of cancer, Cancer Cell 34 (2018) 21–43.

[46] M.B. Sporn, K.T. Liby, NRF2 and cancer: the good, the bad and the importance of context, Nat. Rev. Cancer 12 (2012) 564–571.

[47] H.K. Na, Y.J. Surh, Oncogenic potential of Nrf2 and its principal target protein heme oxygenase-1, Free Radic. Biol. Med. 67 (2014) 353–365.

[48] C.A. Silva-Islas, P.D. Maldonado, Canonical and non-canonical mechanisms of Nrf2 activation, Pharmacol. Res. 134 (2018) 92–99.

[49] K.R. Sekhar, G. Rachakonda, M.L. Freeman, Cysteine-based regulation of the CUL3 adaptor protein Keap1, Toxicol. Appl. Pharmacol. 244 (2010) 21–26.

[50] J. Suh, D.H. Kim, E.H. Kim, S.A. Park, J.M. Park, J.H. Jang, S.J. Kim, H.K. Na, N.D. Kim, N.J. Kim, Y.G. Suh, Y.J. Surh, 15-Deoxy-Δ^{12,14}-prostaglandin J$_2$ activates PI3K-Akt signaling in human breast cancer cells through covalent modification of the tumor suppressor PTEN at cysteine 136, Cancer Lett. 424 (2018) 30–45.

[51] W. Kim, H.N. Lee, J.H. Jang, S.H. Kim, Y.H. Lee, Y.I. Hahn, H.K. Ngo, Y. Choi, Y. Joe, H.T. Chung, Y. Chen, Y.N. Cha, Y.J. Surh, 15-Deoxy-Δ^{12,14}-prostaglandin J$_2$ exerts proresolving effects through nuclear factor E2-related factor 2-induced expression of CD36 and heme oxygenase-1, Antioxid, Antioxid. Redox Signal. 27 (2017) 1412–1431.

[52] N.M. Reddy, H.R. Potteti, S. Vegiraju, H.J. Chen, C.M. Tamatam, S.P. Reddy, PI3K-AKT signaling via Nrf2 protects against hyperoxia-induced acute lung injury, but promotes inflammation post-injury independent of Nrf2 in mice, PLoS One 10 (2015) e0129676.

[53] F. Wang, Y. Xi, W. Liu, J. Li, Y. Zhang, M. Jia, Q. He, H. Zhao, S. Wang, Sanbai melon seed oil exerts its protective effects in a diabetes mellitus model via the Akt/GSK-3β/Nrf2 pathway, J. Diabetes Res. 2019 (2019) 5734723.