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15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 rescues PC12 cells from H_2O_2-induced apoptosis through Nrf2-mediated upregulation of heme oxygenase-1: Potential roles of Akt and ERK1/2

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ABSTRACT

Oxidative stress induced by reactive oxygen intermediates has been implicated in a variety of human diseases including rheumatoid arthritis and neurodegenerative disorders. 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), a terminal dehydration product of prostaglandin D_2, is an endogenous ligand of peroxisome proliferator-activated receptor-γ and exhibits a number of biological activities including the proapoptotic activity. Recent studies have revealed that this cyclopentenone prostaglandin, at non-toxic concentrations, can also exert antiapoptotic or cytoprotective effects. In this study, the underlying mechanisms involved in the protective effects of 15d-PGJ_2 on the H_2O_2-induced cytotoxicty were explored using cultured rat pheochromocytoma (PC12) cells. PC12 cells treated with H_2O_2 underwent apoptosis, which was attenuated by pretreatment with non-toxic concentrations of 15d-PGJ_2. Treatment of the PC12 cells with 15d-PGJ_2 resulted in increased nuclear translocation, DNA-binding and transcriptional activity of NF-E2-related factor 2 (Nrf2), leading to upregulation of heme oxygenase-1 (HO-1) expression, which provided an adaptive survival response against the H_2O_2-derived oxidative cytotoxicity. Transfection of PC12 cells with dominant-negative *Nrf2* gene abolished the 15d-PGJ_2-derived induction of HO-1 expression. Moreover, the 15d-PGJ_2-mediated increases in Nrf2-ARE binding and ARE luciferase activity were suppressed by the dominant-negative mutation as well as the pharmacological inhibition of Akt/protein kinase B or extracellular signal-regulated kinase 1/2 (ERK1/2). Taken together, these findings suggest that 15d-PGJ_2 augments cellular antioxidant defense capacity through activation of Akt and ERK signal pathways that leads to Nrf2 activation, and subsequently HO-1 induction, thereby protecting the PC12 cells from H_2O_2-induced oxidative cell death.

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Abbreviations: ARE, antioxidant response elements; DMEM, Dulbecco's modified Eagle's medium; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; GCL, glutamate-cystein ligase; GSH, reduced glutathione; HO-1, heme oxygenase-1; JC-1, tetraethylbenzimidazolcarbocyanine iodide; MAPK, mitogen-activated protein kinase; NAC, N-acetylcysteine; Nrf2, NF-E2-related factor 2; PARP, poly(ADP-ribose)polymerase; PC12 cells, rat pheochromocytoma cells; PI3K, phosphatidylinositol 3-kinase; PPAR-γ, peroxisome proliferator-activated receptor-γ; PKC, protein kinase C; PVDF, polyvinylidene difluoride membrane; SnPP, tin-protoporphyrin; StRE, stress-response elements; TMRE, tetramethylrhodamine ethyl ester perchlorate; TUNEL, *in situ* terminal nick end-labeling.

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1. Introduction

15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), a representative cyclopentenone prostaglandin, is known to be an endogenous ligand for the peroxisome proliferator-activated receptor-γ (PPAR-γ) [1]. Depending on the cell types and the concentrations used, this cyclopentenone prostaglandin exerts diverse biological activities such as anti-inflammatory, anticarcinogenic, and antioxidant properties [2,3]. However, 15d-PGJ_2 also elicits a cytotoxic effect that is independent of PPAR-γ, and some studies have demonstrated that 15d-PGJ_2 has dual effects, promoting either cell survival or inducing cell death via apoptosis [4–6]. Thus, while 15d-PGJ_2 can provoke the proapoptotic or cytotoxic activity at high concentrations [7,8], at relatively low concentrations, it exetrs cytoprotective or antiapoptotic effects via a mechanism that involves increased biosynthesis of reduced glutathione (GSH) [9,10] and upregulation of some antioxidant enzymes [11,12].

Heme oxygenase-1 (HO-1) is one of the major antioxidant/cytoprotective enzymes that are readily induced in response to oxidative stress. HO-1 catalyzes the rate-limiting step in the heme degradation process, releasing iron, carbon monoxide (CO), and biliverdin. HO-1 expression can be induced not only by its physiological substrate heme but also by a wide variety of noxious stimuli or conditions, such as hyperoxia, hypoxia, proinflammatory cytokines, nitric oxide, heavy metals, UV irradiation, heat-shock, shear-stress, H_2O_2, thiol-reactive substances, etc. [13]. The fact that HO-1 is induced readily by a wide array of toxic insults suggests that HO-1 may function as a critical cytoprotective enzyme [13,14]. It has been recognized that upregulation of HO-1 and subsequent elevation in its catalytic activity could afford cytoprotection [15–18]. Genetic analyses of murine *ho-1* gene have revealed that upregulation of HO-1 expression is modulated by two enhancer sequences (E1 and E2) located at −4 kb (E1) and −10 kb (E2) of the transcriptional start site [19,20]. These E1 and E2 sequences contain repeated stress response elements (StRE), also known as the antioxidant response elements (ARE), which harbor the binding sites for redox-sensitive transcription factors, such as AP-1, NF-κB, NF-E2-related factor 2 (Nrf2), cap'n'collar/basic leucine-zipper proteins, and υ-Maf oncoprotein. Among these transcription factors capable of interacting with ARE, Nrf2 is considered to play the most crucial role in the transcriptional activation of *HO-1* gene expression [21].

15d-PGJ_2 can increase the nuclear translocation of Nrf2 leading to enhanced expression of several phase-II detoxifying enzymes as well as other antioxidant enzymes/proteins [6]. Park et al. [22] have shown that 15d-PGJ_2 promotes activation of Nrf2 through its interaction with PPAR-γ. Alternatively, Itoh et al. [23] demonstrated that 15d-PGJ_2 could stimulate the release of Nrf2 from Keap1 by forming covalently bound adducts with this cytoskeletal protein holding Nrf2 in its inactive state. The resulting free Nrf2 translocates to nucleus, where it binds to ARE in E1 and E2, increasing the expression of HO-1 and peroxyredoxin [23]. However, the upstream signaling that mediates 15d-PGJ_2-induced activation of Nrf2 and subsequent upregulation of HO-1 expression remains largely unresolved.

Here we report that 15d-PGJ_2 activates Nrf2 via the Akt/protein kinase B- and extracellular signal-regulated kinase (ERK)-dependent signal transduction pathways in rat pheochromocytoma (PC12) cells which, in turn, upregulates HO-1 expression, confering protection against the apoptosis induced by H_2O_2.

2. Experimental

2.1. Materials

15d-PGJ_2 was supplied by Cayman Chemical Co. (Ann Arbor, MI, USA). H_2O_2 was purchased from Junsei Chemical Co. (Tokyo, Japan). Poly-D-lysine, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], hemin, and NADPH were the products of Sigma Chemical Co. (St. Louis, MO, USA). Tetraethylbenzimidazolcarbocyanine iodide (JC-1) and tetramethylrhodamine ethyl ester perchlorate (TMRE) were obtained from Molecular Probes, Inc. (Eugene, OR, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, horse serum, F-12 and N-2 supplement factors were provided by Gibco BRL (Grand Island, NY, USA). [γ-^{32}P]ATP was the product of NEN Life Science (Boston, MA, USA). Tin-protoporphyrin (SnPP) was obtained from Alexis (Carlsbad, CA, USA). LY294002 and U0126 were purchased from Calbiochem (San Diago, CA, USA) and TOCRIS (Ellisville, MO, USA), respectively. The luciferase reporter gene fusion constructs for the wild-type ARE and GC mutant ARE were made as described previously [24]. The following oligonucleotides were used (core and mutated sequences are underlined). Wild-type ARE, 5′-CTCAGCCTTCCAAATCG<u>CAGTCA</u>CAG<u>TGACTCAGC</u>AGAATC-3′; GC mutant ARE, 5′-CTCAGCCTTCCAAATCG<u>CAGTCA</u>CAG<u>T-GACTCA</u>**AT**AGAATC-3′. Expression vectors containing dominant-negative mutant of ERK1 and ERK2 (pLCEP4-DN-ERK1 and pLCEP-DN-ERK2) were generous gifts from Dr. Yun-Sil Lee of Korea Institute of Radiological and Medical Science. Full-length Akt and the Akt with K179M mutation (kinase-dead Akt) were provided by Dr. An-Sik Chung of the Korea Advanced Institute of Science and Technology.

2.2. Cell culture

PC12 cells kindly provided by Dr. Young J. Oh (Yonsei University, Seoul, Korea) were maintained in DMEM supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum and cultured at 37 °C in a humidified atmosphere of 10% CO_2 and 90% air. All cells were cultured in poly-D-lysine coated culture dishes. The medium was changed every other day, and the cells were plated at an appropriate density according to the scale of each experiment. After the 24 h subculture, cells were switched to serum-free N-2 defined medium for treatment.

2.3. Measurement of cell viability

PC12 cells were plated at a density of 8×10^4 cells/300 μl in 48-well plates, and the cell viability was determined by using the conventional MTT reduction assay. In the MTT assay, the viable cells convert the cell-permeable soluble dye MTT to the insoluble blue formazan crystals and this reaction is catalyzed by the succinate dehydrogenase, a mitochondrial

respiratory chain enzyme easily inactivated by oxidative stress. After incubation with various chemicals, the cells were treated with MTT solution (1 mg/ml final concentration) for 2 h at 37 °C. The dark blue formazan crystals formed inside the intact mitochondria were solubilized with dimethylsulfoxide, and the absorbance of blue color was measured at 570 nm using a microplate reader (TECAN GmbH, Salzburg, Austria).

2.4. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)

The commercially available *in situ* cell death detection kit (product of Roche Diagnostics, Mannheim, Germany) was used to detect the DNA fragmentation. PC12 cells were fixed by incubation in 10% neutral buffered formalin solution for 30 min at room temperature. Endogenous peroxidase activity was inactivated by 30 min incubation in a methanol solution containing 0.3% H_2O_2 at room temperature and the cells were treated further for 2 min at 4 °C with a permeabilizing solution (0.1% sodium citrate and 0.1% Triton X-100). The cells were then incubated in the TUNEL reaction mixture for 60 min at 37 °C and visualized by confocal microscopy (LEICA TCS NT, Heidelberg, Germany).

2.5. Measurement of mitochondrial transmembrane potential ($\Delta\Psi_m$) depolarization

PC12 cells were plated at a density of 1×10^5 cells/600 μl in a 4-well chamber slide. After incubation with H_2O_2 for 4 h, cells were treated with JC-1 (4 μg/ml) in serum-free N-2 defined medium for 30 min at 37 °C, washed twice with PBS, and examined under a confocal laser scanning microscope. JC-1 fluorescence was monitored either at 530 nm emission as a green fluorescent monomer formed when the $\Delta\Psi_m$ is depolarized or at 590 nm emission as a red fluorescent aggregate formed when the $\Delta\Psi_m$ is hyperpolarized. In both cases, JC-1 was excited at 488 nm. The relative intensity of red to green fluorescence is indicative of $\Delta\Psi_m$ which was quantified using a software provided by LEICA TCS NT. Determination of $\Delta\Psi_m$ depolarization was also carried out using TMRE, a fluorescent dye that accumulates rapidly in mitochondrial compartment due to the large potential difference between cytoplasm and mitochondria. Thus, a decrease in the mitochondrial TMRE fluorescence reflects dissipation of $\Delta\Psi_m$. Following incubation, cells were treated with TMRE (150 nM) for 30 min, rinsed, and examined by confocal microscopy (excitation, 488 nm; emission, 590 nm).

2.6. Western blot analysis

After treatment, the PC12 cells were collected by scraping and centrifugation ($500 \times g$ for 5 min), and the cell pellets were washed and suspended in PBS. After another centrifugation, cells were suspended in lysis-buffer [50 mM Tris–HCl (pH 8.0), 2 mM EDTA, 1% Triton X-100 with a protease inhibitor cocktail tablet] and were subjected to 15-min vigorous shaking at 4 °C. After centrifugation at $12{,}000 \times g$ for 15 min, the protein concentration in the supernatant was determined using the BCA protein assay kit, and then stored at −70 °C. For the Western blot analysis, protein supernatant samples containing protein were electrophoresed on a 12% SDS-polyacrylamide gel, and the separated protein bands were transferred to polyvinylidene difluoride membrane (PVDF) for 2 h at 200 mA. Binding of non-specific proteins was blocked by immersing the blots in a fresh blocking buffer (0.1% Tween-20 in PBS containing 5% non-fat dried milk) for 1 h at room temperature. Subsequently, the blots were incubated with various primary antibodies for 2 h. Dilutions (1:1000) of primary antibodies including anti-Akt, anti-phospho-Akt, anti-poly(ADP-ribose)-polymerase (all are products of Cell Signaling Technology, Beverly, MA, USA), anti-Bax, anti-ERK, anti-phospho-ERK, anti-pp38, anti-Nrf2, anti-HO-1 (products of Santa Cruz Biotechnology, Inc., CA, USA), and anti-actin (Sigma Chemical Co.) were all made in PBS containing 3% non-fat dried milk. Following three washes with PBST (PBS containing 0.1% Tween-20), the blots were incubated for 1 h with secondary antibody conjugated with horseradish peroxidase dissolved in PBS with 3% non-fat dried milk. The blots were washed again three times in PBST and incubated for 1 min in the ECL substrate solution according to the manufacturer's instructions (Amersham Pharmacia Biotech, Arlington Heights, IL, USA) and visualized with X-ray film. The immunoblot analyses were repeated at least three times, and the representative blots were presented.

2.7. Assay for HO activity

The HO activity was measured by spectrophotometric determination of bilirubin formation according to the previously described procedures [25]. Briefly, microsomes obtained from PC12 cells were incubated for 1 h at 37 °C in the dark with hemin (10 μM), NADPH (20 μM), and 1 mg of rat liver cytosol protein which was included as a source of bilirubin reductase. After extraction of incubation mixtures with chloroform, the concentration of bilirubin present in the organic phase was determined from the difference in absorbance between 464 and 530 nm using an extinction coefficient of 40 $mM^{-1}\,cm^{-1}$.

2.8. Assay for caspase-3 activity

The extent of H_2O_2-derived activation of caspase activity in PC12 cells was assessed by using a commercially available colorimetric assay kit according to the protocol supplied by the manufacturer (BioVision, Mountain View, CA, USA). PC12 cells with and without pretreatment with 15d-PGJ_2 for 12 h were plated at a density of 1×10^6 cells/60 mm dish. After treatment with H_2O_2 for 4 h, the cells were harvested by centrifugation. The cell pellets were washed with PBS and lysed by 10 min incubation with 50 μl of chilled lysis-buffer on ice. Lysate was centrifuged at $10{,}000 \times g$ for 1 min at 4 °C and the protein concentration in the supernatant was determined by using the BCA assay. The supernatant was used for the assay of caspase-3 activity by measuring the cleavage of a colorimetric peptide substrate L-aspartic-L-glutamic-L-valyl-L-aspartic acid *para*-nitroaniline (DEVD-*p*NA). Supernatant samples (50 μg) were incubated with the substrate peptide (200 μM) in 50 μl of incubation buffer for 60 min at 37 °C. The production of *para*-nitroaniline was monitored at 405 nm.

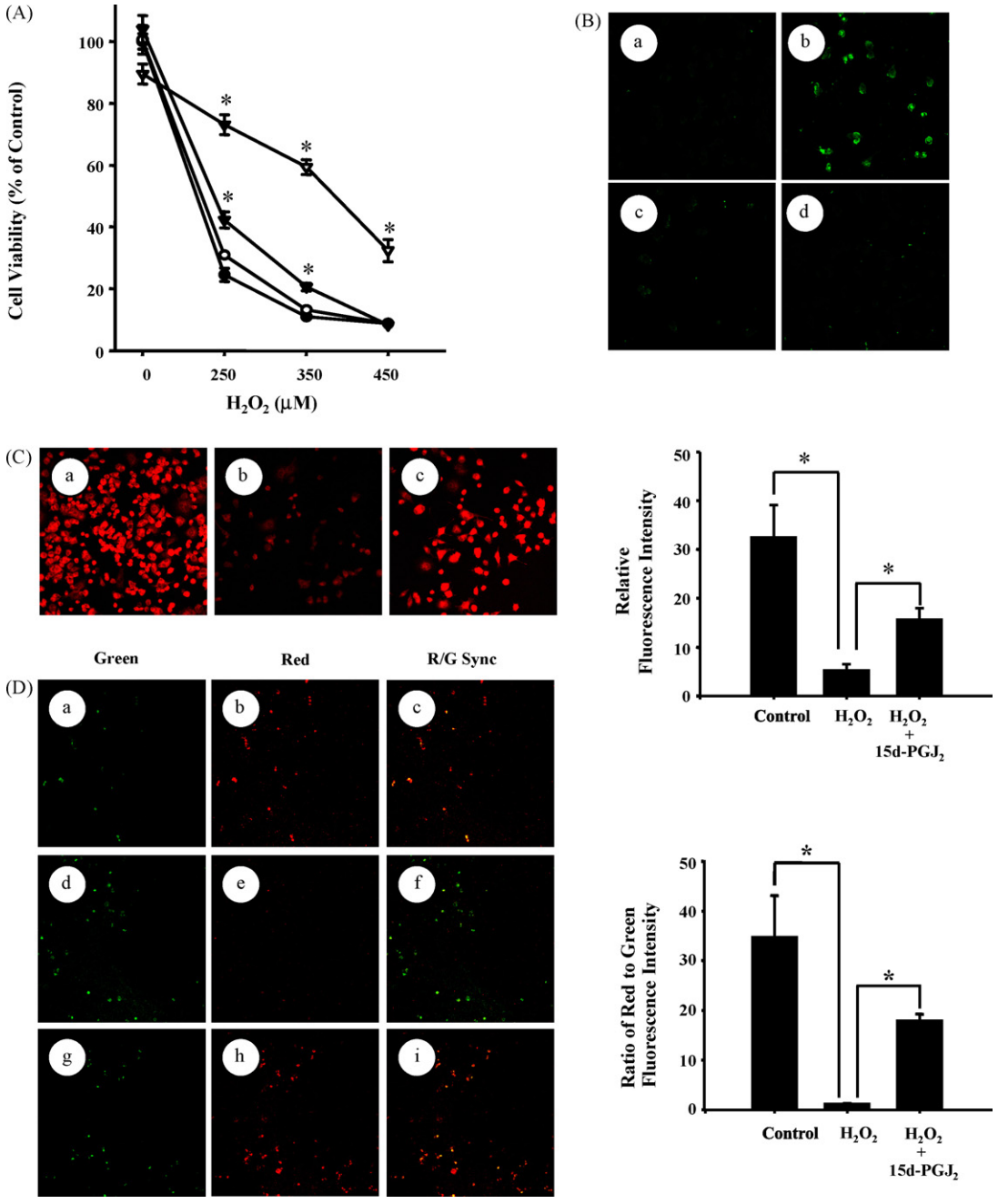

Fig. 1 – Protective effect of 15d-PGJ_2 against H_2O_2-induced cytotoxicity. (A) PC12 cells were treated for 12 h with 0 μM (●), 0.3 μM (○), 1 μM (▼), and 3 μM (▽) of 15d-PGJ_2 followed by exposure to 250 μM H_2O_2 for additional 4 h. The cell viability was determined by the MTT reduction assay. The data are presented as means ± S.D. (n = 3). (*) Significantly different from the H_2O_2 treatment alone ($p < 0.01$). (B) H_2O_2-induced internucleosomal DNA fragmentation was determined by *in situ* terminal nick end-labeling (TUNEL). (a) No treatment; (b) cells exposed to 250 μM H_2O_2 alone for 4 h; (c) cells treated with H_2O_2 for 4 h after 12 h preincubation with 3 μM 15d-PGJ_2; cells treated with 15d-PGJ_2 alone. (C) $\Delta\Psi_m$ was assessed by changes in the TMRE fluorescence as described under Section 2. Images of the cellular fluorescence were acquired by using a confocal laser-scanning microscope. (a) No treatment; (b) cells exposed to 250 μM H_2O_2 alone for 4 h; (c) cells treated with H_2O_2 for 4 h after 12 h preincubation with 3 μM 15d-PGJ_2. $\Delta\Psi_m$ was quantified by measuring average values of the red fluorescence intensity of each image (*$p < 0.01$). (D) Mitochondrial depolarization was confirmed by a decrease in the ratio of red to green fluorescence intensity as measured by JC-1 dye. (a–c) No treatment; (d–f) cells exposed to 250 μM H_2O_2 alone for 4 h; (g–i) cells were treated with H_2O_2 for 4 h after 12 h preincubation with 3 μM 15d-PGJ_2.

2.9. Preparation of nuclear extract proteins

After treatment with 15d-PGJ_2, the PC12 cells (1×10^7 cells/7 ml in 100 mm dish) were harvested, washed with PBS, centrifuged, and resuspended in ice-cold buffer A [10 mM HEPES (pH 7.0), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.2 mM PMSF]. After a 10 min incubation on ice, cells were centrifuged again and resuspended in buffer C [20 mM HEPES (pH 7.9), 20% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT and 0.2 mM PMSF] and incubated for 20 min at 0 °C. After a vortex mixing, the resulting suspension was centrifuged, and the supernatant (nuclear extract) was stored at −70 °C for the Nrf2-ARE binding assay. The protein concentration of the nuclear extract was determined by the Bradford method using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

2.10. Electrophoretic mobility shift assay (EMSA) for determining the ARE binding activity

A synthetic double stranded oligonucleotide containing the Nrf2-binding domain (ARE) was labeled with [γ-^{32}P]ATP using the T4 polynucleotide kinase and separated from the un-incorporated [γ-^{32}P]ATP by gel filtration using a nick spin column (Amersham Pharmacia Biotech, Buckinghamshire, UK). The sequences of double strand oligonucleotides used in the present study were as follows: 5′-TTTTCTGCTGAGT-CAAGGTCCG-3′ and 3′-AAAAGACGACTCAGTTCCAGGC-5′. These oligonucleotides were synthesized by Bionics (Seoul, South Korea). Prior to the addition of ^{32}P-labeled oligonucleotide (100,000 cpm), 10 μg of the nuclear extract was incubated for 15 min on ice in the gel-shift assay binding buffer [20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, and 50 mM Tris–HCl, pH 7.5 with 0.25 μg/ml poly(dI-dC)]. DNA-protein complexes were resolved by 6% polyacrylamide gel electrophoresis carried out at 200 V for 2 h, and this was followed by autoradiography.

2.11. Transient transfection and the reporter gene assay

PC12 cells were seeded at a density of 1×10^5 cells in 6-well plates and grown to 60–70% confluence. For each triplicate of samples, 2.5 μg of the luciferase reporter plasmid construct harboring the ARE promoter (both wild-type and GC mutant) or the equivalent amount of the control plasmid (pTi) was transfected using 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) liposomal transfection reagents (Roche Diagnostics) at the proportion of 1 μg DNA per 5–10 μl DOTAP. CMV-β-galactosidase was used to correct for the transfection efficiency [26]. After the 6 h transfection, cells were treated with vehicle (DMSO) or 15d-PGJ_2, and the cell lysis was carried out using the reporter lysis-buffer. After mixing the cell extract with a luciferase substrate (Promega, Madison, WI, USA), the luciferase activity was determined using a luminometer (AutoLumat LB 953, EG&G Berthold, Bad Widbad, Germany). Luciferase activity was expressed as relative intensity of luminescence ± S.D., which was normalized to β-galactosidase activity.

2.12. Statistical analysis

When necessary, data were expressed as means ± S.D., and statistical analysis for single comparision was performed by using the Student's *t*-test. The criterion employed for statistical significance of difference was $^*p < 0.05$.

3. Results

3.1. 15d-PGJ_2 protected the PC12 cells from H_2O_2-induced cytotoxicity

Pretreatment of PC12 cells with non-toxic concentrations (0.3–3 μM) of 15d-PGJ_2 decreased the H_2O_2-induced toxicity. As shown in Fig. 1A, preincubation with 15d-PGJ_2 for 12 h protected against the H_2O_2-induced cell death in a concentration-dependent fashion as determined by the MTT reduction assay. Cells treated with 250 μM H_2O_2 for 4 h exhibited internucleosomal DNA fragmentation (Fig. 1B) as detected by TUNEL, which is one of the hallmarks of apoptosis. Preincubation with 3 μM 15d-PGJ_2 for 12 h led to a dramatic decrease in the proportion of the TUNEL-positive cells. To further verify the protective effect of 15d-PGJ_2 against the H_2O_2-induced apoptosis, we determined whether the 15d-PGJ_2 could prevent the H_2O_2-induced dissipation of the mitochondrial transmembrane

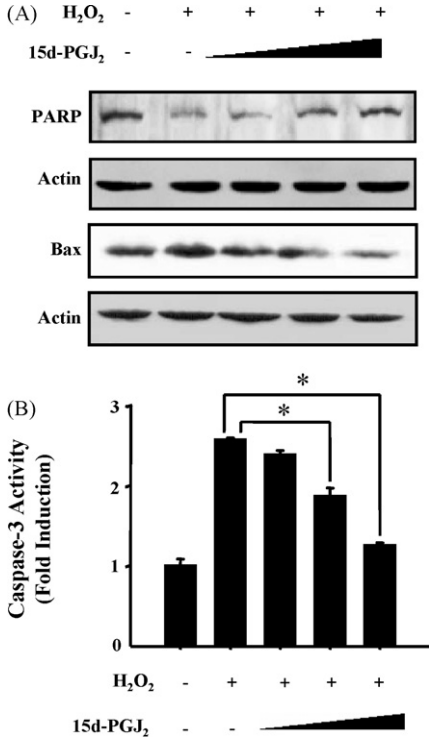

Fig. 2 – Attenuation of H_2O_2-induced apoptotic features by 15d-PGJ_2 (A) PC12 cells were treated with 250 μM H_2O_2 for 4 h after 12 h preincubation with varing amounts (0.1, 1, 3 μM) of 15d-PGJ_2. PARP cleavage and Bax levels were determined by Western blot analysis. (B) Caspase-3/CPP32 activity was measured in terms of cleavage of its substrate DEVD-*p*NA as described in Section 2. (*) Significantly different from the H_2O_2-treated group ($p < 0.01$).

potential ($\Delta\Psi_m$), another general feature of apoptosis. This was measured by using the TMRE fluorescent dye which, after preferential distribution into mitochondria, produces strong red fluorescence. In the PC12 cells treated with H_2O_2, the intensity of mitochondrial TMRE fluorescence decreased, and this decrease was prevented by 15d-PGJ_2 pretreatment (Fig. 1C). Another fluorescent dye (JC-1) was employed to confirm the loss of mitochondrial transmembrane potential caused by exposure to H_2O_2. As a monomer in cytoplasm, JC-1 shows green fluorescence, but when it moves inside the intact mitochondria in response to the large transmembrane potential difference, it aggregates to generate red fluorescence. Thus, the control cells with intact mitochondria can easily be discriminated by its preferential red fluorescence staining. In the cells treated with 250 μM H_2O_2, however, the red staining was replaced by green monomer fluorescence, indicative of mitochondrial depolarization. As in the case with TMRE, in the cells pretreated with 15d-PGJ_2, the loss of JC-1 red fluorescence caused by exposure to H_2O_2 was substantially reduced (Fig. 1D). Actual changes in the mitochondrial transmembrane potential that occurs after the H_2O_2 treatment can be quantified by calculating the relative intensity of red vs. green fluorescence before and after the treatment. Results obtained with JC-1 indicated that the ratio of red to green fluorescence in the H_2O_2-treated cells was decreased, and preincubation of PC12 cells with 15d-PGJ_2 prevented the ratio decrease (Fig. 1D).

Another biochemical hallmark of apoptosis is the cleavage of poly(ADP-ribose)polymerase (PARP) that functions in the repair of damaged DNA. PARP is a 116-kDa nuclear protein that is cleaved specifically to the 85-kDa non-functional fragments by the caspase activity. Treatment of PC12 cells with 250 μM H_2O_2 caused cleavage of PARP, which was mitigated by pretreatment with 15d-PGJ_2 in a concentration-dependent manner (Fig. 2A). Consistent with this finding, the treatment with 250 μM H_2O_2 led to elevated expression of the proapoptotic protein Bax, which was attenuated by pretreatment with 15d-PGJ_2 (Fig. 2A). Furthermore, the caspase-3 activity, which is responsible for the actual execution of apoptosis, was enhanced in the H_2O_2-treated PC12 cells (Fig. 2B). Again, 15d-PGJ_2 pretreatment decreased the H_2O_2-induced enhancement of caspase-3 activity in a concentration-dependent manner.

3.2. 15d-PGJ_2 induced HO-1 expression in PC12 cells

It has been demonstrated that upregulation of HO-1 expression and elevated HO activity play a key role in protecting cells against the toxicity caused by a variety of oxidative insults. In the PC12 cells treated with 15d-PGJ_2, the HO-1 expression was increased in a concentration (Fig. 3A) and time-dependent (Fig. 3B) manner. Likewise, the HO activity was enhanced in proportion to the upregulated HO-1 expression in cells treated with 15d-PGJ_2 (Fig. 3C). In an attempt to determine whether the

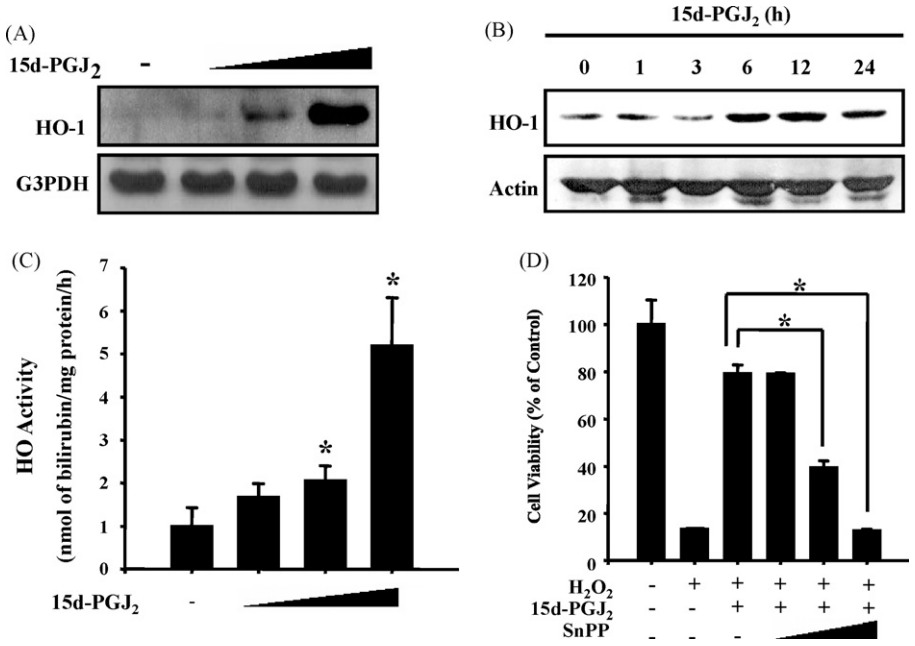

Fig. 3 – Effect of 15d-PGJ_2 on expression and activity of HO-1 in PC12 cells. (A) PC12 cells were treated with 15d-PGJ_2 (0.3, 1, 3 μM) for 12 h. Protein from cell lysates was resolved by SDS-PAGE and subjected to Western blot analysis with HO-1 antibody. G3PDH levels were measured for the confirmation of equal amount of protein loading. (B) PC12 cells were treated with 3 μM 15d-PGJ_2 for the indicated times, and HO-1 expression was determined by immunoblot analysis. Actin levels were measured to ensure equal amounts of protein loading. (C) Cells were treated with increasing concentrations (0.3, 1, 3 μM) of 15d-PGJ_2. Microsomes from cell lysates were incubated with the HO-1 substrate hemin. After incubation, the concentration of bilirubin was determined from the difference in absorbance between 464 and 530 nm using a coefficient of 40 mM^{-1} cm^{-1}. (*) Significantly different from the untreated control group ($p < 0.01$). (D) Cells were preincubated with 3 μM of 15d-PGJ_2 and SnPP (5, 50, 200 μM) for 12 h, followed by exposure to 250 μM H_2O_2. Cytotoxicity was assessed by the MTT reduction assay 4 h after H_2O_2 treatment. The values are the means ± S.D. ($n = 3$). (*) Significantly different between the groups examined ($p < 0.01$).

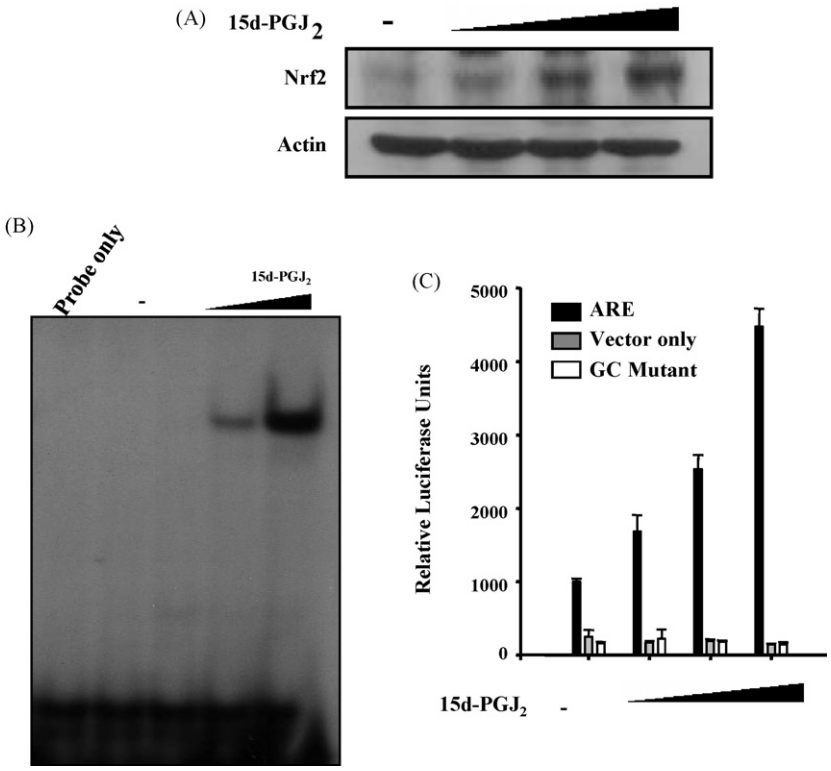

Fig. 4 – Effect of 15d-PGJ_2 on Nrf2 activation in PC12 cells. (A) Nuclear lysates from PC12 cells treated with 15d-PGJ_2 (0.3, 1, 3 μM) for 12 h were probed with a Nrf2 specific antibody. (B) Nuclear extracts were incubated with ^{32}P-labeled oligonucleotides harboring ARE consensus sequence. Nrf2-ARE DNA binding activity was assessed by EMSA as described under Section 2. (C) PC12 cells were cotransfected with wild-type ARE, GC mutant ARE, or control luciferase constructs and CMV-β-galactosidase vector as described in Section 2.1. An appropriate control vector (pTi) was used to ensure specificity. After 6 h, cells were treated with 15d-PGJ_2 (0, 0.3, 1, 3 μM) for additional 12 h and the cell lysate was mixed with a luciferase substrate.

increased HO-1 activity induced by 15d-PGJ_2 is indeed responsible for the cytoprotective effects against the H_2O_2-derived oxidative cell death, we utilized SnPP, an inhibitor of HO activity. As illustrated in Fig. 3D, treatment of PC12 cells with SnPP for 12 h before H_2O_2 challenge attenuated the 15d-PGJ_2-mediated cytoprotection in a concentration-dependent manner. SnPP alone at concentrations used in this experiment did not influence the cell viability (data not shown). These results suggest that the cytoprotective effect elicited by pretreatment with 15d-PGJ_2 is mediated through induction of HO-1 expression.

3.3. *15d-PGJ_2 increased the ARE-Nrf2 binding and ARE-luciferase activity*

The majority of genes encoding xenobiotic detoxifying and antioxidant enzymes have an enhancer region containing ARE [27–29]. Nrf2 is the major transcription factor that binds to ARE and enhances the ARE-driven expression of target genes. Therefore, we first attempted to examine the nuclear accumulation of Nrf2 protein in the 15d-PGJ_2-stimulated PC12 cells. As shown in Fig. 4A, the nuclear levels of Nrf2 were increased by treatment with 15d-PGJ_2 in a concentration-dependent manner. To elucidate the role of Nrf2-ARE binding in the transcriptional activation of *HO-1* gene, EMSA was performed using the oligonucleotides that harbor the HO-1-specific ARE sequence. Incubation of the nuclear extract isolated from 15d-PGJ_2-treated PC12 cells with the [γ-^{32}P]-labeled HO-1 specific ARE oligonucleotides for 1 h resulted in enhanced Nrf2-ARE binding (Fig. 4B). To ensure the 15d-PGJ_2-induced transactivation of Nrf2, PC12 cells were transfected with the luciferase reporter construct containing the wild-type ARE or the construct harboring the mutated ARE sequence in the GC box. As shown in Fig. 4C, the 15d-PGJ_2-driven ARE-luciferase activity was abolished when the ARE sequence was mutated (GC-Mutant).

3.4. *Dominant-negative Nrf2 gene negated 15d-PGJ_2-induced HO-1 expression*

To further explore the role of Nrf2 in upregulating the HO-1 expression, a dominant-negative mutant form of Nrf2 gene was used. Due to the functional inactivation of Nrf2 gene, the PC12 cells harboring dominant-negative mutant form of Nrf2 are expected to be less sensitive to the 15d-PGJ_2 treatment than the normal cells. The PC12 cells transfected with dominant-negative Nrf2 (DN-Nrf2) failed to induce the HO-1 expression (Fig. 5A) and to increase the HO activity (Fig. 5B) in response to 15d-PGJ_2 treatment. Taken together, these findings support that the upregulation of HO-1 elicited by 15d-PGJ_2 is mediated via the Nrf2 activation.

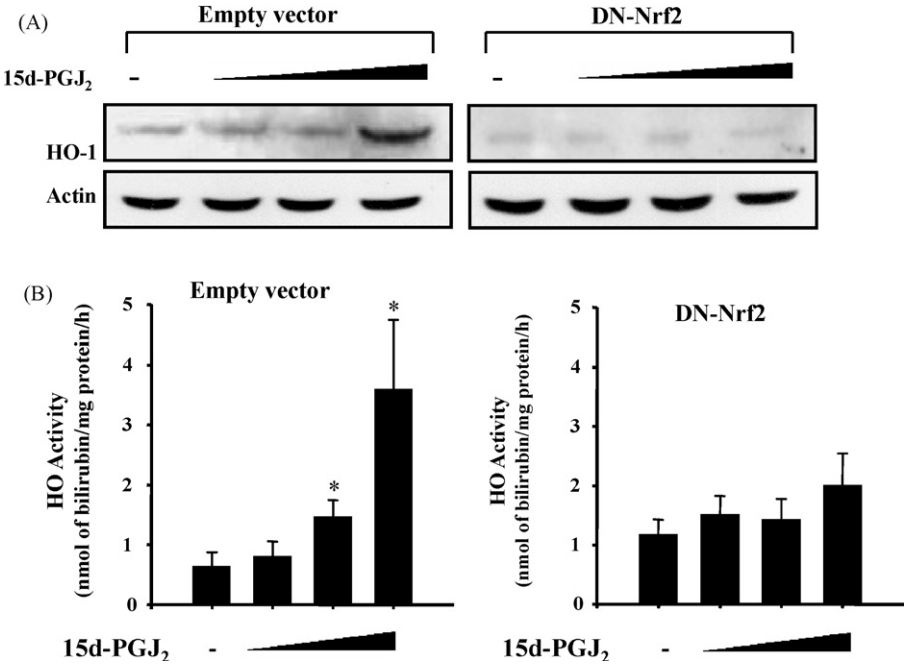

Fig. 5 – Suppression of HO-1 expression in Nrf2 dominant-negative PC12 cells. PC12 cells were transfected with pEF (blank vector) or Nrf2 dominant-negative plasmid DNA (DN-Nrf2) using DOTAP transfection reagent. After 6 h transfection, cells were treated with 15d-PGJ_2 (0.3, 1, 3 μM) for additional 12 h. (A) Effect of DN-Nrf2 transfection on the 15d-PGJ_2-induced upregulation of HO-1 expression. Immunoblots were probed with a HO-1 specific antibody. (B) Effect of DN-Nrf2 transfection on the 15d-PGJ_2-induced HO-1 activity. Microsomes from cell lysates were incubated with the HO-1 substrate hemin. After incubation, the concentration of bilirubin was determined from the difference in absorbance between 464 and 530 nm using a coefficient of 40 mM^{-1} cm^{-1}. (*) Significantly different from the untreated control group ($p < 0.01$).

3.5. *15d-PGJ_2-mediated activation of Akt and ERK signaling*

To elucidate the plausible signal transduction pathways involved in the 15d-PGJ_2-induced Nrf2 activation and HO-1 expression, we examined the phosphorylation of several upstream kinases. Upon 15d-PGJ_2 treatment, concentration- (Fig. 6A) and time- related (Fig. 6B) increases in the phosphorylation of Akt and ERK1/2 were observed in PC12 cells. To determine whether such activation of Akt and ERK1/2 could contribute to the 15d-PGJ_2-mediated protection against the cytotoxic effect of H_2O_2, pharmacological inhibitors of these kinases were utilized. 15d-PGJ_2-mediated cytoprotection against H_2O_2 cytotoxicity was attenuated by LY294002 (Fig. 6C), an inhibitor of phosphatidylinositol 3-kinase (PI3K), upstream of Akt and also by U0126 (Fig. 6D), an inhibitor of MEK1/2, upstream of ERK1/2.

In the following experiments, respective inhibition of Akt and ERK signaling with LY294002 and U0126 attenuated the 15d-PGJ_2-induced increases in both Nrf2-ARE DNA binding activity (Fig. 7A and B) and ARE-driven luciferase activity (Fig. 7C and D). Moreover, pretreatment of PC12 cells with LY294002 or U0126 effectively suppressed the 15d-PGJ_2-mediated upregulation of HO-1 expression (Fig. 7E and F). Likewise, the elevated Nrf2-ARE binding (Fig. 8A) and ARE transactivation (Fig. 8B) in PC12 cells treated with 15d-PGJ_2 were attenuated by the transient transfection with the kinase-dead Akt (KD) or dominant-negative mutant form of ERK (DN ERK1 and DN ERK2).

4. Discussion

Cyclopentenone prostaglandins, particularly those of the J-series, display several unique characteristics. Depending on the concentration applied, they can promote either cell proliferation or apoptotic cell death. Prostaglandin J_2, a dehydration product of prostaglandin D_2, is converted further to 15d-PGJ_2 in a non-enzymatic reaction [30,31]. Due to the presence of two electrophilic α,β-unsaturated carbonyl groups in its cyclopentenone ring, 15d-PGJ_2 can readily form thiol-conjugates with reduced GSH and the cysteine residues contained in many intracellular proteins [5,6,32,33]. Recently, it has been demonstrated that pre-exposure of cells to non-toxic concentrations of 15d-PGJ_2 can protect against the damages caused by oxidative and nitrosative stress. In this connection, 15d-PGJ_2 has been shown to induce the expression of phase II detoxifying or antioxidant enzymes such as the glutathione *S*-transferase [4,11,34] and glutamate-cystein ligase (GCL) [9,10].

HO-1, also known as heat-shock protein 32 (HSP32) or inducible HO, is a 32-kDa protein transiently activated by a wide variety of noxious stimuli including oxidative and nitrosative stress that can cause GSH depletion. Upregulation of HO-1 leading to elevation of HO activity has been shown to provide neuroprotective effects by converting the pro-oxidant heme to biologically active antioxidant by-products such as biliverdin/bilirubin and also to heme inactivating carbon monoxide. The transgenic mice overexpressing HO-1 in the

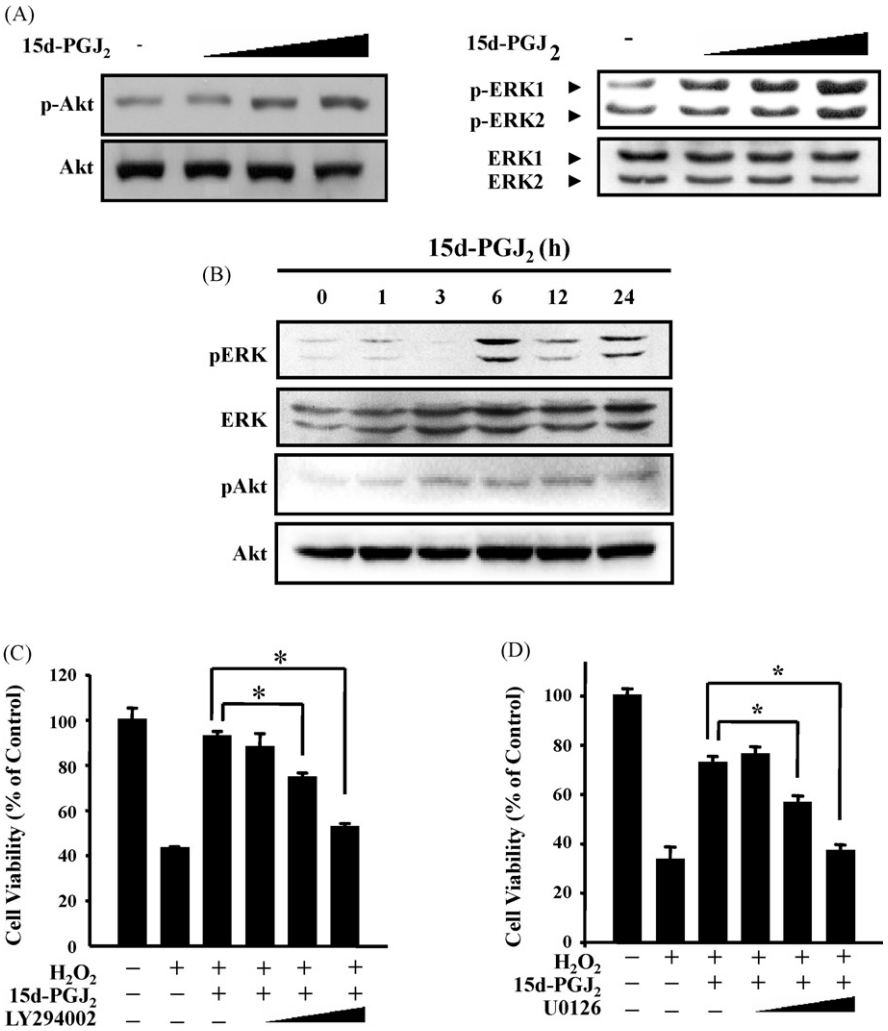

Fig. 6 – 15d-PGJ_2-induced phosphorylation of ERK and Akt in PC12 cells. (A) PC12 cells were exposed to 15d-PGJ_2 (0.1, 1, 3 μM) for 12 h. Protein from cell lysates was resolved by SDS-PAGE and analyzed by Western blot using phospho-Akt and phospho-ERK 1/2 antibodies. (B) PC12 cells were treated with 15d-PGJ_2 (3 μM) for the indicated durations, and levels of phosphorylated as well as total ERK and Akt were measured by Western blot analysis. (C) Effect of a pharmacological inhibitor of PI3K (LY294002) on 15d-PGJ_2-mediated cytoprotection against H_2O_2 toxicity. Cells were preincubated with 3 μM of 15d-PGJ_2 and LY294002 (5, 20, 50 μM) for 12 h, and then exposed to 250 μM H_2O_2 for additional 4 h. (D) Effect of a pharmacological inhibitor of MEK1/2 (U0126) on 15d-PGJ_2-mediated cytoprotection against H_2O_2 toxicity. The concentrations of U0126 used were 5, 15, 25 μM. Cytotoxicity was assessed by the MTT reduction assay. The values are the means ± S.D. ($n = 3$). (*) Significantly different between the groups examined ($p < 0.01$).

brain attenuated neuronal cell injury caused by ischemic stroke through overproduction of cGMP, upregulation of Bcl-2 expression, inhibition of nuclear localization of p53, and suppression of lipid peroxidation [35]. HO-1 overexpressing cells derived from transgenic mice or the cells treated with a HO-1 inducer are relatively resistant to oxidative or nitrosative stress [36–38].

In the present study, we note that 15d-PGJ_2 pretreatment rescues the PC12 cells from the H_2O_2-induced toxicity via upregulation of HO-1 expression. In line with our findings, Lin et al. have demonstrated that neuronal cells originating from rat and human tissues pretreated with 15d-PGJ_2 at low concentrations had elevated HO-1 expression and survived H_2O_2-induced apoptosis [39]. Similarly, prior induction of HO-1 in RAW264.7 mouse macrophages by 15d-PGJ_2 was shown to attenuate cell death caused by diesel exhaust particle extracts [40]. While 15d-PGJ_2 has been reported to induce HO-1 expression in various cell types [12,23,39–45], the underlying regulatory signaling pathways are not fully clarified.

In the human hepatoma (HepG2) cell line exposed to thiol antioxidants and reducing agents, the HO-1 induction by 15d-PGJ_2 was attenuated, suggesting that the cellular thiol status is critical for the upregulation of HO-1 expression elicited by 15d-PGJ_2 [44]. In human lympocytes, 15d-PGJ_2-induced expression of HO-1 mRNA and protein was inhibited by *N*-acetylcystein (NAC) and other antioxidants [43]. Similarly, 15d-PGJ_2-induced HO-1 expression in the mouse hepatoma (Hepa) cell line was abrogated by exposure to NAC [40]. In contrast, the expression

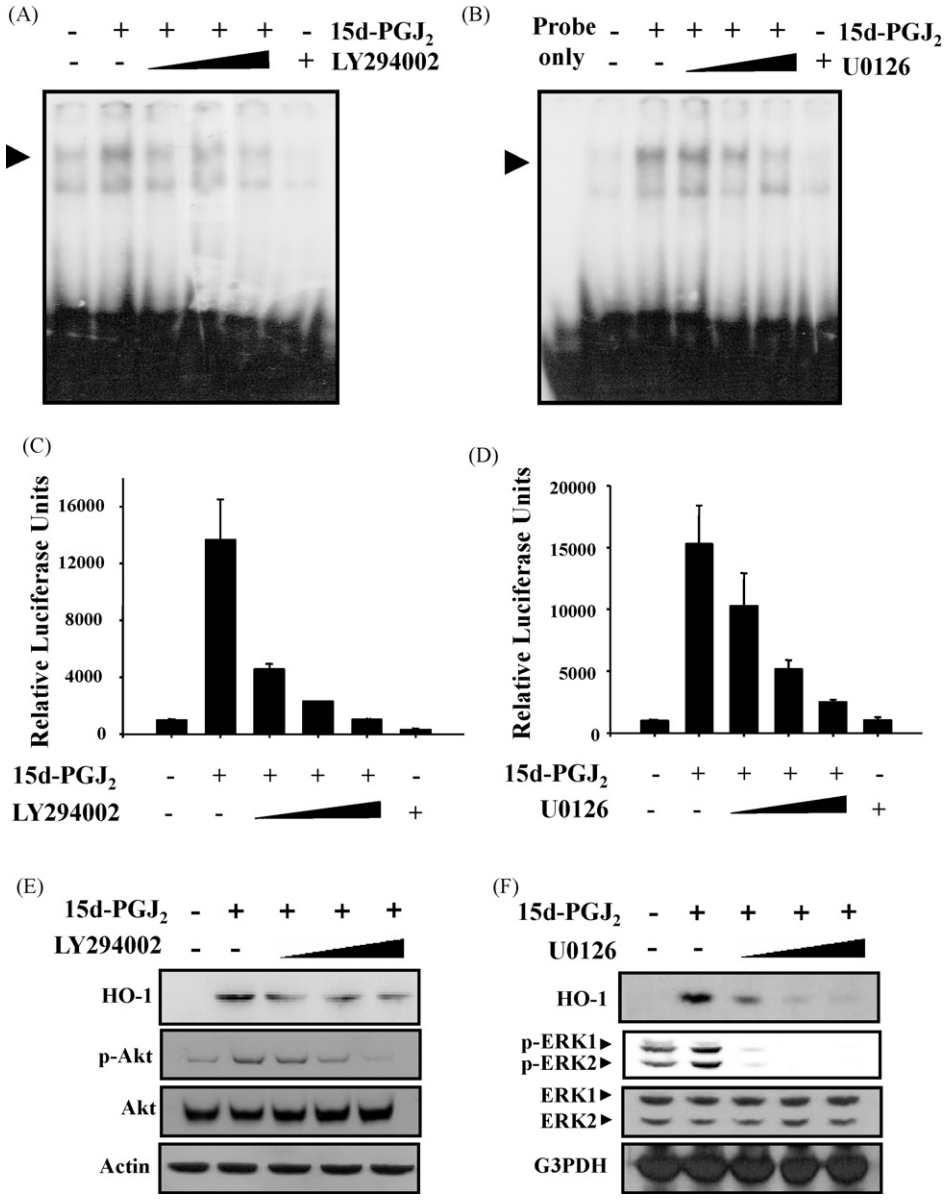

Fig. 7 – Attenuation of Nrf2-ARE binding, Nrf2 transcriptional activity and HO-1 expression by LY294002 or U0126. Cells were pretreated for 1 h in the absence and presence of LY294002 (5, 20, 50 μM) or U0126 (5, 15, 25 μM), and then incubated for an additional 6 h with 15d-PGJ_2 (3 μM). (A and B) Effects of LY294002 (A) and U0126 (B) on 15d-PGJ_2-induced Nrf2-ARE binding activity. (C and D) Effect of LY294002 (C) and U0126 (D) on 15d-PGJ_2-mediated transcriptional activation of Nrf2. (E and F) Effect of LY294002 (E) and U0126 (F) on the 15d-PGJ_2-induced HO-1 expression in PC12 cells.

of HO-1 inducible with 15d-PGJ_2 was enhanced by pretreatment with an inhibitor of GSH synthesis or transition metal ions [43].

The central sensor of intracellular oxidative stress is the cytosolic Keap1-Nrf2 complex. In response to oxidative stress, Nrf2 is released from Keap1 and transmits the stress signal to the nucleus for activation of distinct set of genes encoding phase II detoxifying enzymes as well as several stress-responsive proteins including HO-1 [46,47]. As demonstrated with the rat hepatocyte cell line [22], we note that 15d-PGJ_2 induces the release of Nrf2 from Keap1 and promotes the nuclear translocation of Nrf2 in PC12 cells, leading to the upregulation of HO-1 expression. 15d-PGJ_2 may facilitate the release of Nrf2 from Keap1 by several distinct mechanisms. Chen et al. reported that 15d-PGJ_2 treated to PC12 cells at sublethal concentrations significantly increased the mRNA expression as well as the enzyme activity of GCL, the rate-limiting enzyme of GSH synthesis [10]. 15d-PGJ_2-induced expression of GCL was mediated, in part, by Nrf2. The expression of both catalytic and regulatory subunits of GCL peaked approximately 6 h after 7.5 μM 15d-PGJ_2 treatment. In this study, GCL upregulation was preceded by Nrf2 nuclear accumulation in as early as 3 h. We also confirmed that the treatment of PC12 cells with 3 μM 15d-PGJ_2 induced expression of GCLC which appeared to occur after HO-1 upregulation (data not shown).

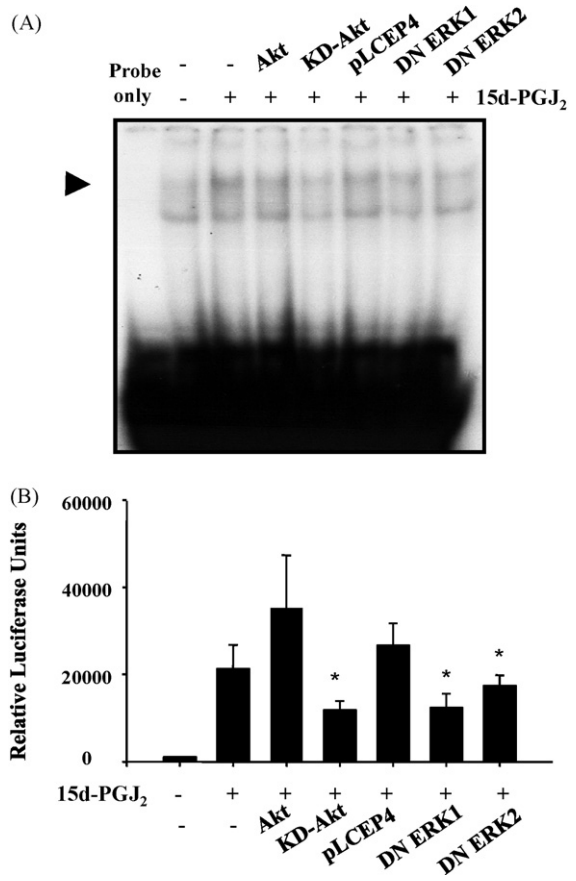

Fig. 8 – Effects of transfecting kinase-dead Akt or dominant-negative ERK1/2 on the 15d-PGJ$_2$-induced increases in Nrf2-ARE binding (A), and Nrf2 transcriptional activity (B) PC12 cells were co-transfected with full-length Akt (Akt), kinase-dead Akt (KD), dominant-negative mutant ERK1/2 (DN-ERK1/2) or control vector (pCEP4) and cMV-β-galactosidase, and then exposed to 3 μM 15d-PGJ$_2$ for 6 h. CMB-β-galactosidase was used to correct for the transfection efficiency. The ARE binding and transcriptional activity of Nrf2 were determined by EMSA and the luciferase reporter gene assay respectively, as described in Section 2.1. The values are the means ± S.D. (n = 3).

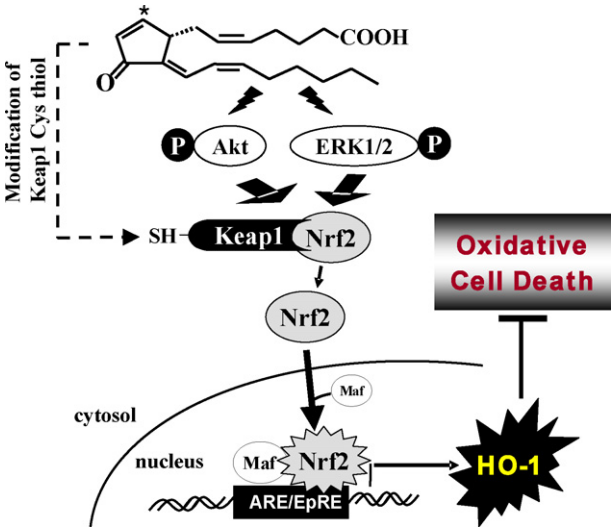

Fig. 9 – Proposed mechanisms for the induction of HO-1 in 15d-PGJ$_2$-treated PC12 cells. 15d-PGJ$_2$ can stimulate PI3K-Akt and ERK signaling through phosphorylation. It may also modify Keap1 cysteine thiol(s) through Michael addition. Both events facilitate the dissociation of Nrf2 from Keap1 and its nuclear translocation for binding to ARE/EpRE, leading to induction of HO-1 expression. Asterisk denotes the electrophilic center that can covalently modify Keap1 cysteine.

15d-PGJ$_2$ that contains an α,β-unsaturated carbonyl moiety was reported to form Michael reaction adducts with cysteine residues present in Keap1, which facilitates the dissociation of Nrf2 from Keap1, leading to nuclear translocation of Nrf2 for induction of HO-1 and *PrxI* gene expression [23]. The two electrophilic carbonyl groups located in the cyclopentenone ring structure of 15d-PGJ$_2$ are anticipated to form covalent adducts with cysteine containing proteins like Keap1 [5,6]. This may lead to a relatively rapid induction of Nrf2.

In this study, we also note that 15d-PGJ$_2$ induces Nrf2 activation in PC12 cells through PI3K or ERK signaling, which may represent another plausible mechanism responsible for Nrf2 activation (Fig. 9). Recent studies suggest that the activation of Nrf2 occurs by a coordinated process which involves different signaling upstream kinases such as protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and PI3K. Phosphorylation of Nrf2 by PKC promoted its dissociation from Keap1, whereas the Nrf2-S40A mutant with Ser residue in amino acid 40 replaced by Ala remained sequestered with Keap1, suggesting that PKC-catalyzed phosphorylation of Nrf2 at Ser-40 is a critical signaling event leading to ARE-mediated cellular antioxidant response [48]. Shen et al. [49] have shown that the transcriptional activity of Nrf2 transactivation domain was stimulated by ERK and JNK signaling pathways while the p38 MAPK plays a negative role. In IMR-32 and SH-SY5Y human neuoblastoma cells, *tert*-butylhydroquinone- and hemin-induced activation of Nrf2 and subsequent expression of ARE-driven antioxidative proteins were inhibited by PI3K inhibitors such as LY294002 and wortmannin [50,51]. Pretreatment of hepatocellular carcinoma HepG2 cells with MAPK inhibitors, such as PD98059 and SB202190, reduced the pyrrolidine dithiocarbamate-induced nuclear translocaion of Nrf2 and subsequent expression of GCL indicating that MAPK-directed phosphorylation is required for the activation of Nrf2 [52,53]. However, in the same cell line, functional inactivation of ERK by PD98059 or dominant-negative mutation failed to block 15d-PGJ$_2$-induced HO-1 expression [44], which is contradictory to our observation with PC12 cells. The 5′-flanking region of the human HO-1 gene was found to contain several sequences that could serve as potential regulatory elements involving transcription factors other than Nrf2 [54]. These include AP-1, NF-κB, STAT binding sequences, etc. Moreover, a putative StRE, responsible for induction of HO-1 gene by 15d-PGJ$_2$ in mice, has been reported to be lacking in the human HO-1 gene

[44]. As HepG2 cells are human origin, it might be possible that HO-1 upregulation by 15d-PGJ_2 in these cells might be mediated by transcription factors other than Nrf2. These results suggest that unlike the PC12 cells originating from rats, Nrf2-ARE signaling in HepG2 cells may not play a key role in mediating the induction of HO-1 gene by 15d-PGJ_2.

In summary, 15d-PGJ_2 induced the upregulation of HO-1 expression through activation of Nrf2, which confers protection against the H_2O_2-induced apoptotic death in PC12 cells. While some studies suggested an association between 15d-PGJ_2-induced HO-1 upregulation and Nrf2 activation [23,40], few have revealed the involvement of upstream signaling pathways mediating the activation of Nrf2 that leads to ARE binding. One of the most salient features of our present study is that PI3K-Akt and ERK1/2 are involved in HO-1 induction via Nrf2 activation in the 15d-PGJ_2-stimulated cells. Thus, pharmacological and genetic inhibition of ERK1/2 or PI3K suppressed Nrf2 activation and subsequent HO-1 expression. Results of our study imply the potential involvement of these upstream kinases in Nrf2 activation and HO-1 upregulation by 15d-PGJ_2. However, the complete molecular milieu that links all these events needs to be elucidated. Continued attempts to identify novel target molecules responsible for the HO-1 regulation and to clarify their cross talk with upstream and downstream signaling molecules will pave the way to exploiting preventive and/or therapeutic strategies for the management of oxidative stress-mediated disorders.

Acknowledgements

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