

Inhibitive effects of 15-deoxy- $\Delta^{12,14}$ -prostaglandin J_2 on hepatoma-cell proliferation through reactive oxygen species-mediated apoptosis

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Objective: 15-Deoxy- $\Delta^{12,14}$ -prostaglandin J_2 (15d-PGJ₂) induces reactive oxygen species (ROS)-mediated apoptosis in many malignant cells, which has not been studied in hepatoma cells. In this study, we investigated whether 15d-PGJ₂ induced apoptosis in hepatocellular carcinoma (HCC) associated with ROS.

Materials and methods: The LM3, SMMC-7721, and Huh-7 HCC cell lines were treated with 15d-PGJ₂ (5–40 μ M) for 24, 48, and 72 hours. Cholecystokinin 8 was used to detect the cytotoxicity of 15d-PGJ₂. Flow cytometry, Hoechst staining, and Western blotting were used to analyze apoptosis. ROS were combined with the fluorescent probe dihydroethidium and then observed by fluorescence microscopy and flow cytometry. Activation of JNK and expression of Akt were detected by Western blotting.

Results: 15d-PGJ₂ inhibited HCC cell proliferation and induced apoptosis in a dose- and time-dependent manner. Apoptosis was mainly induced via an intrinsic pathway and was ROS-dependent, and was alleviated by ROS scavengers. ROS induced JNK activation and Akt downregulation in HCC cells.

Conclusion: 15d-PGJ₂ induced ROS in HCC cell lines, and inhibition of cell growth and apoptosis were partly ROS-dependent.

Keywords: 15-deoxy- $\Delta^{12,14}$ -prostaglandin J_2 , hepatoma cell, ROS, apoptosis, JNK

Introduction

On account of its gradually increased incidence and high recurrence rate, hepatocellular carcinoma (HCC) has become the third-most frequent cancer and leading cause of cancer death worldwide.^{1–3} Surgery is currently the preferred and most effective treatment; however, the prognosis remains poor.^{4,5} Chemotherapeutic agents that are active against liver cancer are constantly being developed and tested in order to improve the survival rate of patients with HCC, but their effect is still unsatisfactory.^{6,7}

15-Deoxy- $\Delta^{12,14}$ -prostaglandin J_2 (15d-PGJ₂) is a dehydrated product of prostaglandin D_2 . It was originally discovered as an endogenous lipid mediator and ligand for the nuclear receptor PPAR γ , which regulates lipid and carbohydrate metabolism, adipocyte differentiation, and inflammation.^{8,9} Early studies indicated that 15d-PGJ₂ can inhibit cancer cell proliferation and induce apoptosis through a PPAR γ -dependent pathway.^{10–12} However, recent evidence indicates that the induction of apoptosis and inhibition of cancer cell proliferation by 15d-PGJ₂ are attributable to generation of reactive oxygen species (ROS) rather than activation of PPAR γ .^{13–15} 15d-PGJ₂ contains an α,β -unsaturated carbonyl group that acts as an electrophilic center, and thus it can react with and modify nucleophiles, such as glutathione and thioredoxin, resulting in

alteration of the cellular redox status.^{16–18} The accumulation of oxidized proteins induced by 15d-PGJ₂ also increases cellular oxidative stress.^{19,20}

ROS, including superoxide anion, hydrogen peroxide, and hydroxyl radical, are produced from normal oxygen metabolism. In normal cells, excessive ROS can be eliminated by a series of potent antioxidants, thus ROS can be maintained at low concentrations, which exerts their biological activities.²¹ By activating several pathways, such as p53 proapoptotic signaling and c-Jun N-terminal kinase (JNK), and regulating phosphorylation and ubiquitination of Bcl-2 family members, ROS induced apoptosis.^{22–24}

In the present study, we investigated whether 15d-PGJ₂ induced ROS and ROS-mediated apoptosis in the LM3, Huh-7, and SMMC-7721 HCC cell lines. We confirmed that 15d-PGJ₂-induced ROS in HCC cell lines and inhibition of cell growth, as well as apoptosis, were partly ROS-dependent.

Materials and methods

Cell lines and culture

All experiments were performed in accordance with ethical standards and in compliance with the Declaration of Helsinki, as per national and international guidelines. This study was approved by the Ethics Committee of Tongji University. Three HCC cell lines – LM3, Huh-7, and SMMC-7721 – and normal hepatocytes (LO2) were purchased from the Cell Bank of the Chinese Academy of Sciences Committee Type Culture Collection (Shanghai, People's Republic of China [PRC]). All four cell lines were cultured in high-glucose Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (GE Healthcare, Little Chalfont, UK), 100 U/mL penicillin, and 100 µg/mL streptomycin (Thermo Fisher Scientific) in a 5% CO₂ and 95% air incubator at 37°C.

Reagents

15d-PGJ₂ (dissolved in methyl acetate) and *N*-acetylcysteine (NAC) were purchased from Sigma-Aldrich (St Louis, MO, USA). The cell-counting kit (CCK-8) used was produced by Dojindo (Dojindo Laboratories, Kumamoto, Japan). The antibodies used in this study included those directed against proliferating cell nuclear antigen (PCNA; Proteintech, Chicago, IL, USA), cytochrome C (Cell Signaling Technology, Danvers, MA, USA), Bax (Cell Signaling Technology), caspase 3 (Cell Signaling Technology), caspase 9 (Proteintech), caspase 8 (Proteintech), PARP1 (Proteintech), Akt (Proteintech), p-Akt (Cell Signaling Technology), JNK (Proteintech), p-JNK (Cell Signaling Technology), and β-actin (Proteintech). An annexin V–fluorescein isothiocyanate (FITC) and propidium

iodide (PI) apoptosis-detection kit was purchased from BD Biosciences (San Jose, CA, USA). The ROS fluorescent probe dihydroethidium (DHE) was purchased from Beyotime Biotechnology (Shanghai, People's Republic of China).

Cell proliferation and viability

The LM3, SMMC-7721, and Huh-7 HCC cell lines were plated at 3×10⁴ cells/mL in 96-well plates (100 µL medium per well). After being seeded for 24 hours, all wells were treated with 15d-PGJ₂ (diluted with phosphate-buffered saline [PBS] to final concentrations of 5, 10, 20, 30, and 40 µM); each concentration was incubated with five replicate samples. Cell proliferation and viability were measured using the CCK-8 assay and a microplate reader (Synergy H4; BioTek Instruments Inc, Winooski, VT, USA) at a wavelength of 450 nm after treatment for 24, 48, and 72 hours. Half-maximal inhibitory concentration (IC₅₀) values were calculated by using CalcuSyn Version 2.0 software (Biosoft, Cambridge, UK).

Apoptosis analyses

The HCC cells were first plated into six-well plates at 10⁶ cells/mL, exposed to 10, 20, and 30 µM 15d-PGJ₂ for 48 hours, and analyzed with flow cytometry and Hoechst staining.

Flow cytometry

The cells were collected after trypsin treatment for digestion and washed twice with PBS. The processed cells were added to Falcon tubes at 10⁶ cells/mL containing 100 µL 1× binding buffer and incubated for 20 minutes at room temperature with annexin-V/FITC supplemented with PI. Cells were considered to be apoptotic if they were either annexin V⁺/PI⁻ (early apoptotic) or annexin V⁺/PI⁺ (late apoptotic). The rate of apoptosis was measured by flow cytometry within 1 hour.

Hoechst 33342 staining

The cells in six-well plates treated with 15d-PGJ₂ at designed concentrations for 48 hours were washed with PBS and fixed in 4% paraformaldehyde for 10 minutes. The fixed cells were then washed with PBS and perfused with Hoechst 33342 stain (1 µL added to 200 µL PBS). The filled six-well plates were placed at 4°C in the dark for 20 minutes. Fluorescence microscopy (Leica Microsystems, Wetzlar, Germany) was used to examine the blue fluorescent cells.

Western blotting

Cells treated with 15d-PGJ₂ (10, 20, and 30 µM) for 48 hours were collected after trypsin digestion and washed twice with PBS. Radioimmunoprecipitation-assay lysis buffer with protease inhibitors and phenylmethanesulfonyl fluoride were

added to the cell suspensions to lyse the cells and collect cellular extracts. The concentration of the prepared protein was determined by the bicinchoninic acid protein assay (Thermo Fisher Scientific). The cellular proteins were then mixed with 5× sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample-loading buffer and boiled for 10 minutes. The treated samples were subjected to SDS-PAGE following standard protocols. Nonspecific binding was blocked with 5% nonfat powdered milk (dissolved in PBS) for 2 hours, and blots were incubated overnight at 4°C with the following antibodies: PCNA (1:500), cytochrome C (1:500), Bax (1:1,000), caspase 3 (1:200), caspase 9 (1:500), caspase 8 (1:500), Akt (1:1,000), JNK (1:500), p-JNK (1:500), p-Akt (1:500), and β-actin (1:1,000). The next day, all the membranes were washed three times with PBS containing 0.1% Tween 20 before incubation with the secondary antibody (antirabbit or antimouse IgG [1:2,000]) for 1 hour at room temperature. Finally, membranes were washed with PBS containing 0.1% Tween 20 three times for 5 minutes each, and proteins were detected by fluorescence using the Odyssey two-color infrared laser imaging system (Li-Cor Biosciences, Lincoln, NE, USA).

Measurement of ROS

The HCC cells were first plated into six-well plates at 10⁶ cells/mL, exposed to 10, 20, and 30 μM 15d-PGJ₂ for 4 hours, or pretreated with NAC for 1 hour. The fluorescent probe DHE (dissolved in dimethyl sulfoxide) was added to the medium at a final concentration of 50 μM. The processed cells were incubated at 37°C for 1 hour, avoiding light, and washed twice with PBS before observation under fluorescence microscopy. For flow-cytometry analysis, the processed cells were collected for suspension in PBS and washed with 1 mL cold PBS. ROS were detected at a wavelength of 590–610 nm, and the cells were divided into two subsets.

Statistical analysis

All experimental data were evaluated by calculating means ± standard deviation analyzed by SPSS 20.0 software (IBM, Armonk, NY, USA). Student's *t*-test and one-way analysis of variance, followed by Tukey's test when *F* was significant, were performed to compare the results of the CCK-8 assay. In all comparisons, *P*<0.05 was considered statistically significant. The bar charts were obtained using GraphPad Prism for Windows version 5.0 (GraphPad Software Inc, La Jolla, CA, USA).

Results

15d-PGJ₂ inhibits HCC cell growth

Proliferation of all three cell lines (LM3, MMC-7721, and Huh-7) after treatment with 15d-PGJ₂ (5, 10, 20, 30, and

40 μM) for 24, 48, and 72 hours was examined using the CCK-8 assay. The cell-viability curve was constructed according to OD, as shown in Figure 1A–D. 15d-PGJ₂ inhibited HCC cell growth in a dose- and time-dependent manner. IC₅₀ values were calculated according to the results of CCK8 assays. IC₅₀ values for these cell lines were 15.5 (LM3), 18.7 (SMMC-7721), and 36.7 μM (Huh-7). PCNA was detected by Western blotting (Figure 1E).

15d-PGJ₂ induces apoptosis in HCC cell lines

Flow cytometry, Hoechst staining, and Western blotting were used to investigate whether 15d-PGJ₂ induced apoptosis in HCC cell lines. Flow cytometry showed that the percentage of early and late apoptotic cells was significantly higher after treatment with 15d-PGJ₂ for 48 hours than in the normal culture-group cells (Figure 2A). Besides, it also showed the difference between the HCC cell lines and normal hepatocytes (LO2), which served as a control for the apoptosis assays (Figure 2B). After 15d-PGJ₂ treatment, all cells were stained with Hoechst 33342, which revealed that the DNA differed in shape and showed high intensity in fluorescence compared with the normal group (Figure 2C). Western blotting demonstrated apoptosis-related protein expression, including cytochrome C, Bax, caspase 3, caspase 9, caspase 8, and PARP1, in all HCC cell lines. Figure 2D shows the significant change in all detected proteins except caspase 8, which indicated that the apoptosis induced by 15d-PGJ₂ was mainly dependent on the intrinsic apoptosis pathway.

15d-PGJ₂ induces ROS generation in HCC cell lines

ROS generated after 15d-PGJ₂ treatment were observed and measured using fluorescence microscopy and flow cytometry, respectively. DHE-probe staining of ROS-positive cells showed increased fluorescence intensity (Figure 3A). The ratio of ROS-positive to normal cells was analyzed by flow cytometry. ROS generation was shown to be dependent on treatment time with 15d-PGJ₂ (Figure 3B).

15d-PGJ₂ induces ROS-mediated JNK activation and downregulation of Akt pathway

ROS are suggested to be strong activators of JNK, leading to mitochondrial cytochrome C release, caspase 9 activation, and initiation of the intrinsic apoptosis pathway. JNK activation was detected by Western blotting. Figure 4A shows that phosphorylation of JNK was increased in all three cell lines after 15d-PGJ₂ treatment for 48 hours when compared

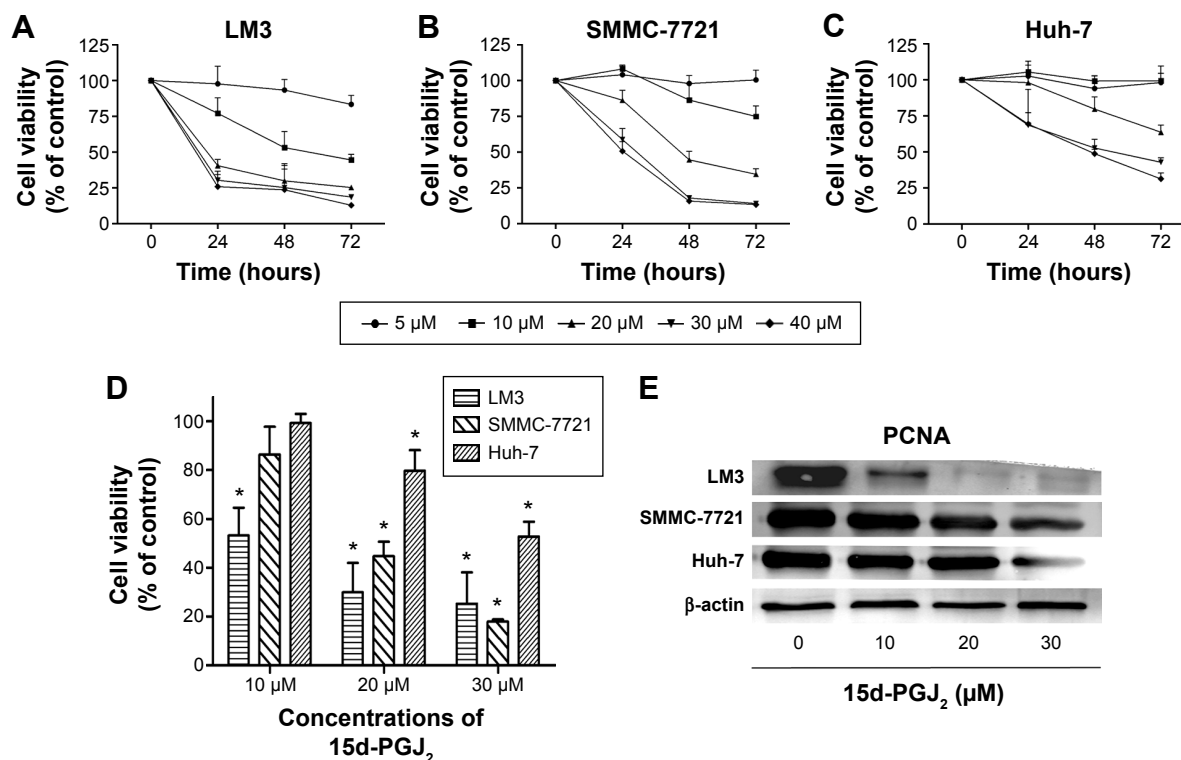


Figure 1 Effects of 15d-PGJ₂ on HCC cell proliferation.

Notes: (A–C) LM3, SMMC-7721, and Huh-7 cells treated with 15d-PGJ₂ (5, 10, 20, 30, and 40 μ M) for 24, 48, and 72 hours. Cell viability expressed as a percentage of viability under controlled culture conditions according to Cell Counting Kit 8 assay results (n=5). 15d-PGJ₂ showed cytotoxic activity in HCC cells in a dose- and time-dependent manner. (D) Cell viability of LM3, SMMC-7721, and Huh-7 cells treated with 15d-PGJ₂ (10, 20, and 30 μ M) for 48 hours, analyzed in a histogram (**P*<0.05 for control culture vs 15d-PGJ₂). (E) PCNA expression was detected after 15d-PGJ₂ treatment (10, 20, and 30 μ M) for 48 hours in all three HCC cell lines.

Abbreviations: 15d-PGJ₂, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂; HCC, hepatocellular carcinoma; PCNA, proliferating cell nuclear antigen.

to the normal group. Expression of phosphorylated Akt was decreased by 15d-PGJ₂. ROS-mediated JNK activation, cytochrome C release, and apoptosis induction, as well as downregulation, were alleviated by the appearance of the ROS scavenger NAC.

Discussion

The present study confirmed ROS generation by 15d-PGJ₂ in HCC cell lines. The apoptosis induced by 15d-PGJ₂ in HCC cells was partly dependent on ROS, and was alleviated by the ROS scavenger NAC. These results are in accordance with previous studies of 15d-PGJ₂ in other cell lines, from which we determined the dose of drug to use.^{13,25}

We showed that 15d-PGJ₂ exerted cytotoxic activity and inhibited proliferation in all HCC cell lines. According to the CCK-8 assay, 15d-PGJ₂ inhibited proliferation of LM3 and SMMC-7721 cells in a time- and dose-dependent manner, and to a lesser extent in Huh-7 cells. After 48 hours' treatment with 20 μ M 15d-PGJ₂, cell viability was reduced to approximately 25%, 50%, and 75% in LM3, SMMC-7721, and Huh-7, respectively, and we chose the three effective concentrations (10, 20, and 30 μ M) for our continuing

experiments (Figure 1D). PCNA is an auxiliary protein of DNA polymerase, which is related to DNA synthesis in eukaryotic cell DNA and reflects the state of cell proliferation. It was detected by Western blotting to corroborate the growth inhibition induced by 15d-PGJ₂, and the result was consistent with the CCK-8 assay.²⁶

15d-PGJ₂ has been reported to induce apoptosis in many malignant cells, including hepatoma cells.^{10,13,14,25} In the present study, flow cytometry and Hoechst 33342 staining were used to detect and analyze the number and state of apoptotic cells. The increase in the proportion of early and late apoptotic cells and metamorphic DNA confirmed that the HCC cell death induced by 15d-PGJ₂ was mainly via apoptosis, in accordance with previous studies.^{13,25} Apoptosis-related proteins were investigated using Western blotting. The increased expression of cleaved caspase 3 and cleaved PARP1 indicated apoptosis activation and DNA damage in 15d-PGJ₂-treated HCC cell lines. The expression of proteins of the intrinsic apoptosis pathway, including cytochrome C, Bax, and caspase 9, was also increased relevantly to the concentration of 15d-PGJ₂. Caspase 8, the key enzyme activated in the extrinsic apoptosis pathway, was not significantly affected. These data indicate

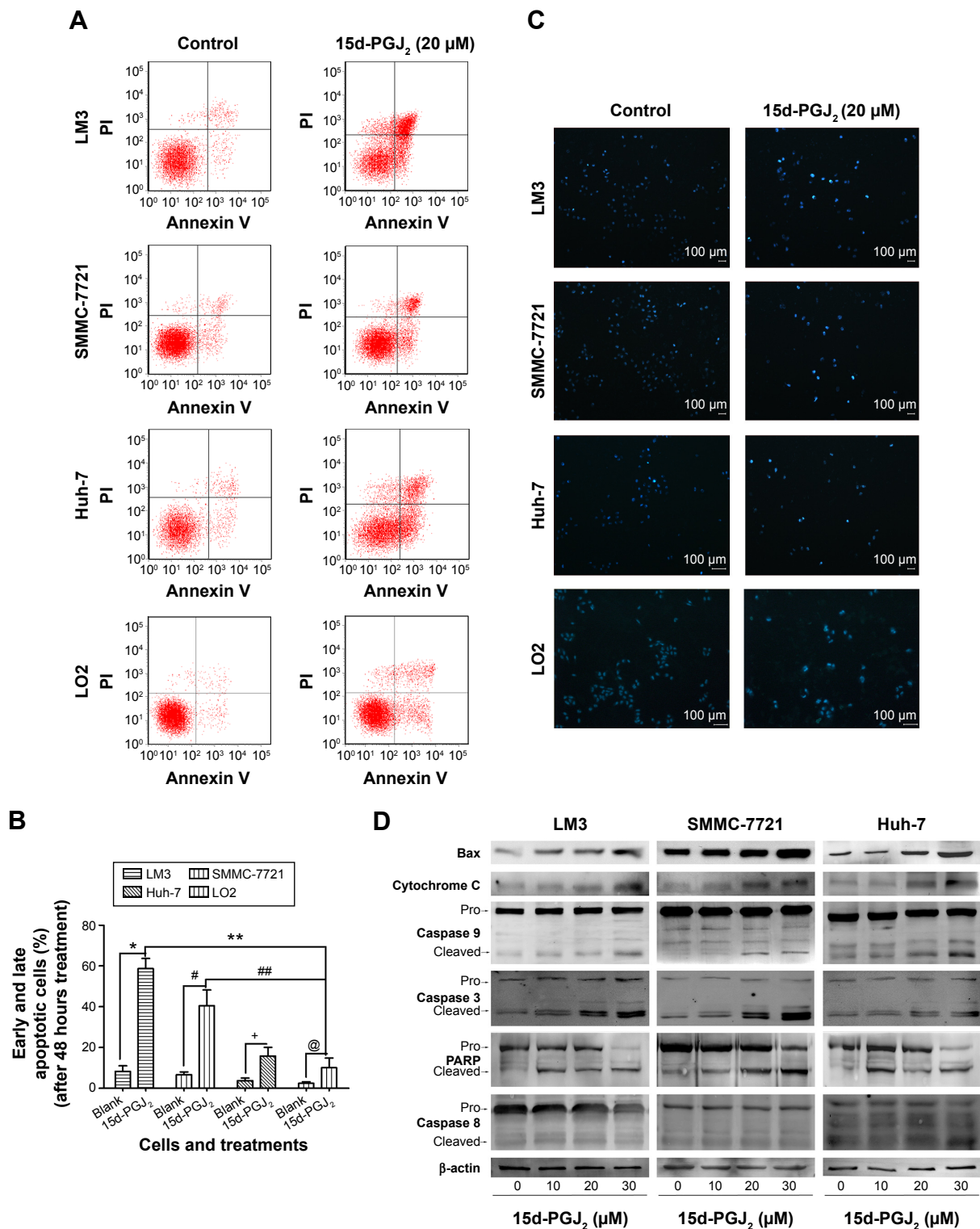


Figure 2 15d-PG₂ induces apoptosis in hepatoma cells.

Notes: (A) LM3, SMMC-7721, Huh-7, and LO2 cells treated with 15d-PG₂ (20 μM) for 48 hours and then stained with annexin V-FITC and PI to detect apoptosis by using flow cytometry. Cells were considered to be apoptotic if they were either annexin V⁺/PI⁻ (early apoptotic) or annexin V⁺/PI⁺ (late apoptotic). The percentage of early and late apoptotic cells was counted at the end of 48 hours' treatment in each cell line and the difference compared with normal culture-group cells (B). This also showed the difference between the HCC cell lines with normal hepatocytes (LO2), which served as a control for the apoptosis assays (**P*<0.01 for LM3 cells in control culture vs 20 μM 15d-PG₂; #*P*<0.01 for SMMC-7721 cells in control culture vs 20 μM 15d-PG₂; **P*<0.05 for Huh-7 cells in control culture vs 20 μM 15d-PG₂; @*P*<0.05 for LO2 cells in control culture vs 20 μM 15d-PG₂; ***P*<0.01 for LM3 cells vs LO2 cells, both in 20 μM 15d-PG₂; ###*P*<0.01 for SMMC-7721 cells vs LO2 cells, both in 20 μM 15d-PG₂; n=3). (C) Hoechst 33342 staining of LM3, SMMC-7721 and Huh-7 cells (light blue represents malformed cell nuclei, white bar for 100 μm). (D) Cytochrome C, Bax, pro- and cleaved caspases, including caspase 3, caspase 9, and caspase 8, and PARP1 of three HCC cells were detected by Western blot.

Abbreviations: 15d-PG₂, 15-deoxy-Δ^{12,14}-prostaglandin J₂; PI, propidium iodide; HCC, hepatocellular carcinoma; vs, versus.

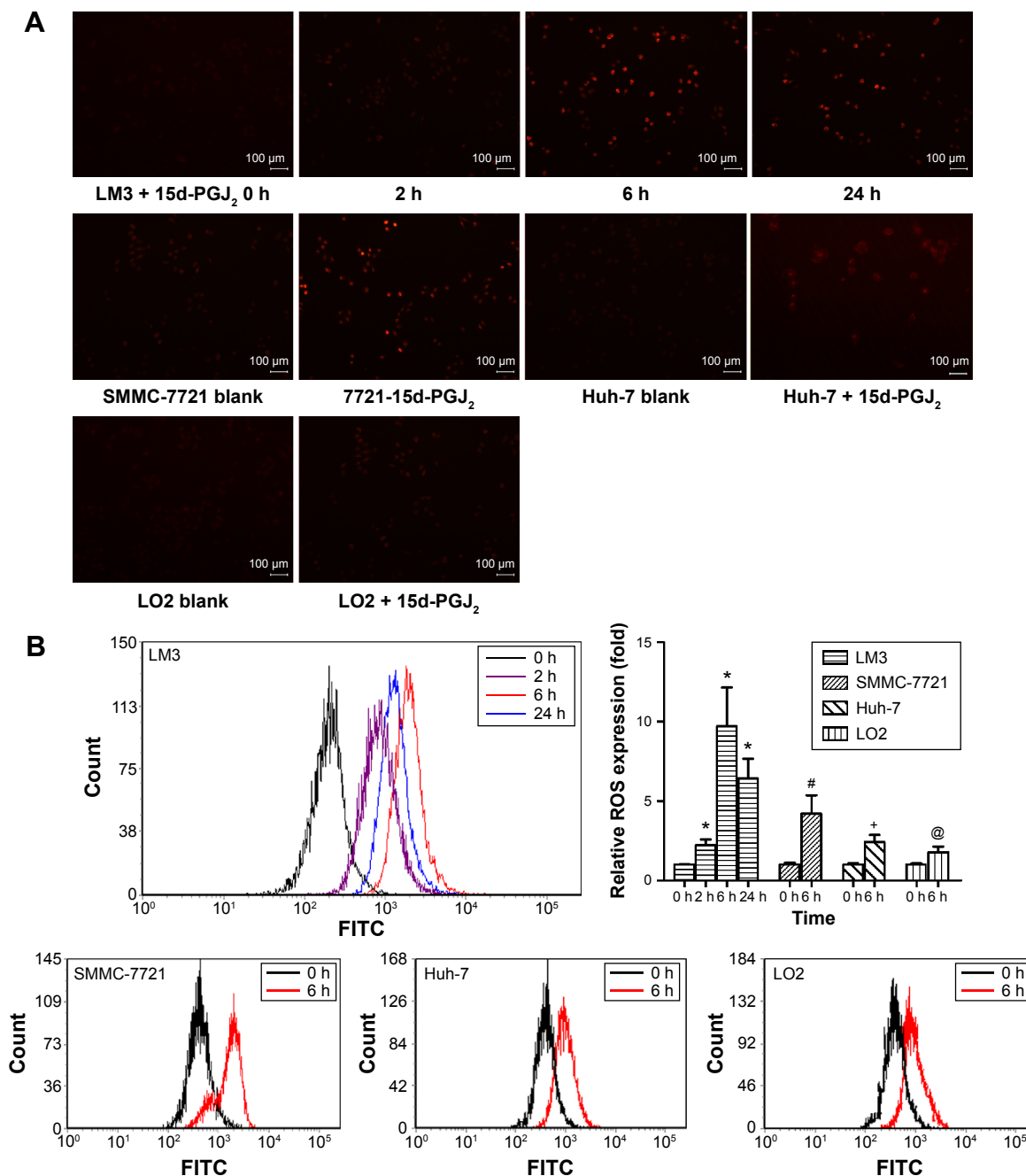


Figure 3 15d-PGJ₂ induces ROS generation in HCC cell lines.

Notes: (A) Cells were incubated with the ROS fluorescent probe DHE, then detected by fluorescence microscopy (bright red represents ROS-positive cells; control culture vs 20 μM 15d-PGJ₂ in LM3 cells for 2, 6, and 24 hours; control culture vs 20 μM 15d-PGJ₂ in SMMC-7721 and Huh-7 cells for 6 hours), and (B) flow cytometry and analysis in histogram with corresponding time points (black lines represent the detected time after incubation for 0 hours, purple line for 2 hours, red lines for 6 hours, and blue lines for 24 hours (**P*<0.05 for control culture vs 15d-PGJ₂ in LM3, #*P*<0.05 for control culture vs 15d-PGJ₂ in SMMC-7721, +*P*<0.05 for control culture vs 15d-PGJ₂ in Huh-7, @*P*<0.05 for control culture vs 15d-PGJ₂ in LO2).

Abbreviations: 15d-PGJ₂, 15-deoxy-Δ^{12,14}-prostaglandin J₂; ROS, reactive oxygen species; HCC, hepatocellular carcinoma; DHE, dihydroethidium; FITC, fluorescein isothiocyanate; h, hours; vs, versus.

that apoptosis induced by 15d-PGJ₂ is mainly dependent on the intrinsic but not extrinsic apoptosis pathway, which is consistent with Date et al, who used the caspase 3 inhibitor Z-DEVD-FMK to block apoptosis of human hepatoma cells that had been treated with 15-d-PGJ₂.¹⁰

The ROS concentration in cancer cells is higher than in normal cells, due to their unique increased metabolic properties.^{27,28} Elevated levels of ROS in cancer cells promote carcinogenesis and cancer progression by angiogenesis.^{29,30} However, it has been shown that excess ROS beyond the antioxidant

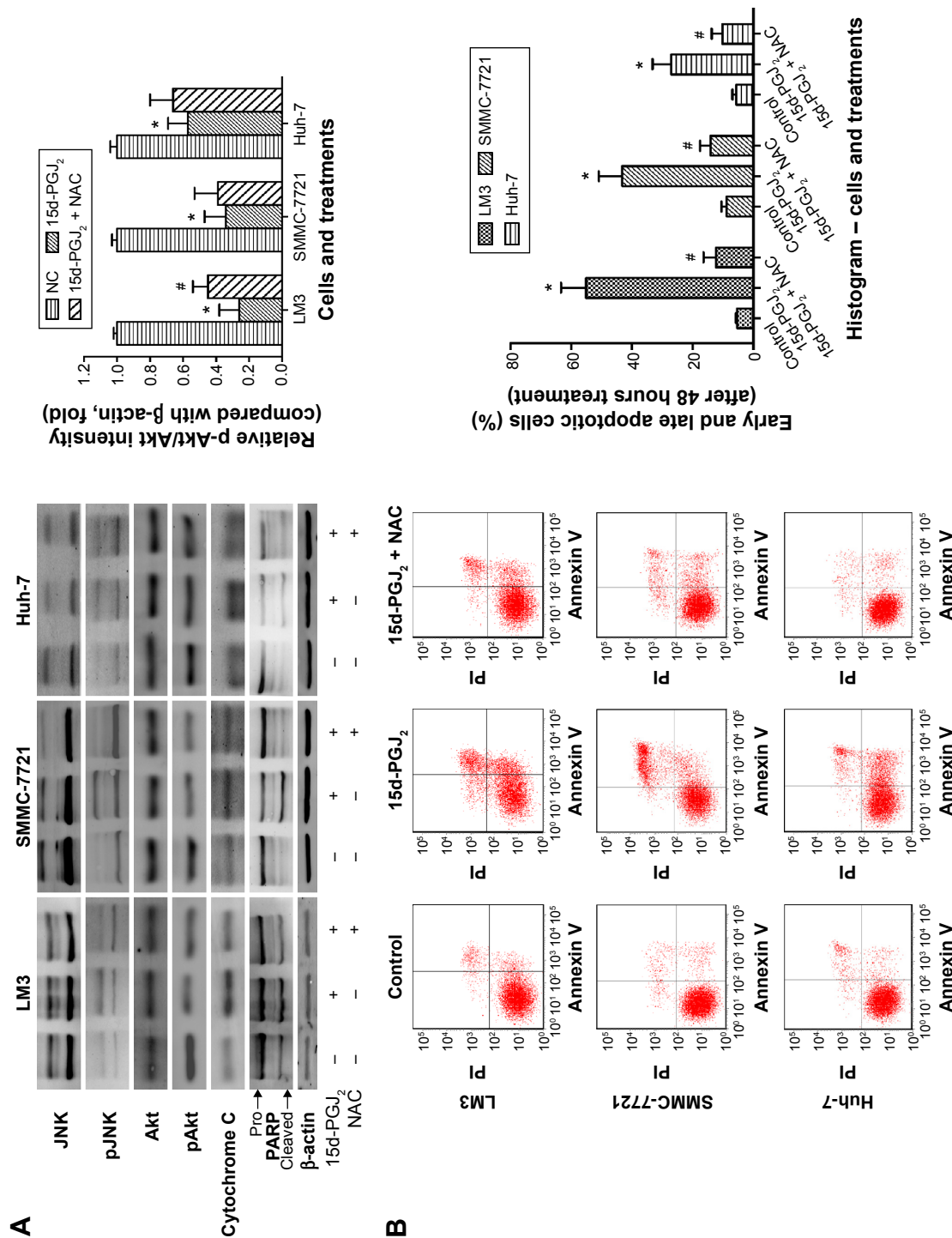


Figure 4 Effects of 15d-PG₂ on HCC are ROS-dependent.

Notes: (A) Western blotting analysis of all three HCC cell lines treated with 15d-PG₂ (20 μ M) for 48 hours in the absence or presence of NAC pretreatment (2 mM) for 1 hour using antibodies against JNK, p-JNK, Akt, p-Akt, cytochrome C, and PARP. A reduction of the ratio of p-Akt and Akt indicated downregulation of the Akt pathway after 15d-PG₂ treatment, and the effect was alleviated in the presence of NAC ($^{*}P < 0.05$ for control culture vs 15d-PG₂ in LM3, SMMC-7721, and Huh-7 cell lines; $^{*}P < 0.05$ for 15d-PG₂ + NAC vs 15d-PG₂ in LM3). (B) Flow-cytometry analysis of all three cell lines at baseline or treated with 15d-PG₂ (20 μ M) with or without NAC (2 mM) for 48 hours ($^{*}P < 0.05$ for control culture vs 15d-PG₂; $^{*}P < 0.05$ for 15d-PG₂ vs 15d-PG₂ + NAC).
Abbreviations: 15d-PG₂, 15-deoxy- $\Delta^{2,14}$ -prostaglandin J₂; HCC, hepatocellular carcinoma; ROS, reactive oxygen species; NAC, N-acetylcysteine; PI, propidium iodide; vs, versus.

capacity of the cells can induce apoptosis.^{31,32} The present study revealed that the level of ROS generation is related to the treatment time of 15d-PGJ₂. ROS were detected at 2 hours after 15d-PGJ₂ treatment; the level increased up to 6 hours, and was still detectable after 24 hours by flow cytometry. NAC pretreatment increased the cell viability and decreased expression of cytochrome C and cleaved PARP, indicating that ROS generation is critical in 15d-PGJ₂-induced HCC cell apoptosis (Figure 4). Although both the result of CCK-8 assays and flow cytometry showed that 15d-PGJ₂ showed an obvious effect on cell-proliferation inhibition and cell-apoptosis promotion in all three cell lines, the difference of the effect among the cell lines was also big. According to our results, 15d-PGJ₂ also had a different effect on ROS production in these cell lines, which is consistent with the trend of the apoptosis and cell viability. Therefore, we speculate that the different sensitivity to ROS of different hepatoma cell lines may count for the different effect under the 15d-PGJ₂ treatments.

The activation of JNK, a potent proapoptotic kinase, is thought to be one the main factors induced by excess ROS.^{13,23} Phosphorylation of JNK was detected by Western blotting in 15d-PGJ₂-treated cells compared with normal cells, and decreased when NAC was present (Figure 4A). These data suggest that ROS-mediated JNK activation contributes to apoptosis induced by 15d-PGJ₂. We also observed down-regulation of the Akt pathway, which is one of the most effective antiapoptotic pathways.³³ The decreased expression of phosphorylated Akt signifies the weakened antiapoptotic pathway and reduces inhibition of JNK, which in turn increases apoptosis (Figure 4B).

As mentioned earlier, 15d-PGJ₂ contains an α,β -unsaturated carbonyl group that can act as an electrophilic center and allow itself to exert biological actions beyond some of the receptor-dependent pathways, such as inhibition of the NF κ B pathway, disruption of the Keap1–Nrf2 complex, and alteration of cellular redox status.³⁴ Some studies have shown the other possible pathways by which 15d-PGJ₂ can exert anticancer activity.^{35–37}

Conclusion

In summary, this study verifies that 15d-PGJ₂ can inhibit HCC cell proliferation and induce apoptosis in a dose- and time-dependent manner. We also demonstrated that 15d-PGJ₂ induces ROS production and ROS-mediated apoptosis, as well as JNK activation and Akt downregulation in hepatoma cells.

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Disclosure

The authors report no conflicts of interest in this work.

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