RESEARCH ARTICLE

15d-PGJ2 Induces Apoptosis of MCF-7 and MDA-MB-231 Cells via Increased Intracellular Calcium and Activation of Caspases, Independent of ERα and ERβ

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Abstract

Reports indicate that 15-deoxy-delta-12,14-prostaglandin-J2 (15d-PGJ2) has anticancer activities, but its mechanisms of action have yet to be fully elucidated. We therefore investigated the effects of 15d-PGJ2 on the human breast cancer cell lines, MCF-7 (estrogen receptor ERα+/ERβ+) and MDA-MB-231 (ERα-/ERβ+). Cellular proliferation and cytotoxicity were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays while apoptosis was determined by fluorescence microscopy and flow cytometry using annexin V-propidium iodide (PI) staining. ER expression was determined by Western blotting. Intracellular calcium was stained with Fluo-4 AM while intracellular caspase activities were detected with Caspase-FLICA® and measured by flow cytometry. We showed that 15d-PGJ2 caused a significant increase in apoptosis in MCF-7 and MDA-MB-231 cells. ERα protein expression was reduced in treated MCF-7 cells but pre-incubation with the ERα inhibitor 'ICI 182 780' did not affect the percentage of apoptotic cells. The expression of ERβ was unchanged in both cell lines. In addition, 15d-PGJ2 increased intracellular calcium (Ca^{2+}) staining and caspase 8, 9 and 3/7 activities. We therefore conclude that 15d-PGJ2 induces caspase-dependent apoptosis that is associated with an influx of intracellular Ca^{2+} with no involvement of ER signaling.

Keywords: 15d-PGJ$_2$, MCF-7, MDA-MB-231, apoptosis, estrogen receptor, intracellular calcium, caspase

Introduction

15-deoxy-delta-12,14-prostaglandin-J2 (15d-PGJ$_2$) is formed from the final dehydration product of prostaglandin-D2 into J series prostanoids characterized by the presence of an α,β-unsaturated ketone in the cyclopentenone ring)(Egger et al., 2015). It is a potent endogenous ligand for peroxisome proliferator-activated-receptor γ (PPARγ), a member of the nuclear receptor superfamily of ligand-dependent transcription factors, and serves as a direct inhibitor of several other signal transduction pathways (Zingarelli et al., 2003; Yen et al., 2014). It has broad effects on various cellular systems eg., cell growth, differentiation and immune response (Croasdell et al., 2015; Kim et al., 2015), and the mechanism of action could be PPARγ-dependent or -independent (Kim et al., 2003; Li and Pauza, 2009; Napimoga et al., 2013; Mughal et al., 2015).

Growing evidences show that 15d-PGJ$_2$ plays a role as an anticancer agent. Many have reported on the anti-proliferative effects and induction of apoptosis by 15d-PGJ$_2$ on cancer cells such as on leukemic, colorectal (Shin et al., 2009; Dionne et al, 2010), lung, renal (Fujita et al., 2012), gastric (Chen et al., 2003), prostate and bladder carcinoma cell lines (Chaffer et al., 2006). Our group previously showed that the anticancer effects of 15d-PGJ$_2$ are the results of PPARγ-independent event via perturbation of the mitochondrial membrane potential (Yaacob et al., 2013). To further understand its anticancer mechanism, the current study focused on the involvement of the estrogen receptor (ER), in 15d-PGJ$_2$-induced apoptosis in ERα+/ERβ+ and ERα-/ERβ+ breast cancer cell lines, MCF-7 and MDA-MB-231, respectively. Changes in intracellular calcium (Ca^{2+}) staining as well as caspase-driven apoptosis were also investigated. We showed herein that the 15d-PGJ$_2$-induced apoptosis is caspase-dependent and is associated with an increase in the intracellular Ca^{2+}. Our results also indicate no influence of ER signaling on the observed apoptotic activity.

Materials and Methods

Cell culture and treatment

Human breast cancer cell lines, MCF-7 and MDA-MB-231, were purchased from American Type Culture Collection (ATCC, USA). The cells were maintained in

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Roswell Park Memorial Institute Medium (RPMI) 1640-no phenol red, supplemented with 10 % charcoal-stripped fetal bovine serum (FBS) and 1 % penicillin-streptomycin solution (Gibco, USA) at 37 °C in 5 % CO_2. MCF-7 and MDA-MB-231 cells were seeded in appropriate culture vessels at a density of $2x10^5$ and $1x10^6$ cells/ml, respectively, until 70 % confluence prior to treatments. The cells were then treated for 24 h with 50 % effective concentrations (EC_{50}) of 15d-PGJ$_2$ (15 and 10 µM, respectively). The stock solution of 15d- PGJ$_2$ (Cayman Chemicals, USA) was prepared in DMSO and diluted fresh prior to experiments.

Determination of EC_{50} concentration of 15d-PGJ$_2$

Cell cytotoxicity was measured based on cellular release of lactate dehydrogenase (LDH) into the culture medium using the LDH Detection Kit (Roche Diagnostics, Germany) according to the manufacturer's protocol. The cells were seeded in 24-well cell culture plates and treated with five different concentrations of 15d-PGJ$_2$ (5-25 µM). Following incubation (6-72 h), cell-free supernatants were transferred to 96-well plates for determination of LDH activity. The reaction was stopped by the addition of 1 M hydrochloride and the absorbance was read at 429 nm using a microplate reader (SpectraMax, Molecular Devices, USA). The reference wavelength was set at 620 nm. The % cytotoxicity was calculated based on the formula:

$$\text{Cytotoxicity} = \frac{\text{Experimental value} - \text{Low control}}{\text{High control} - \text{Low control}}$$

The concentration of 15d-PGJ$_2$ that caused 50 % cell death was considered as the EC_{50}.

Determination of cell proliferation

The 3-(4,5-dimethylthiazol-2-y(1)-2,5-diphenyltetrazolium bromide (MTT) assay ISigma-Aldrich, USA) was used to determine cellular proliferation following treatment with EC_{50} 15d-PGJ$_2$ and 100 nM ICI 182 780 (Tocris Bioscience, UK) (Popolo et al., 2011) for 72 h. Cells were seeded at $1x10^4$ per well in a 96-well plate and incubated at 37°C in humidified atmosphere with 5 % CO_2. Ten µl MTT (5 mg/ml) was added to each well and the cells were further incubated for 4 h. The medium was removed and 100 µl DMSO was added to each well to dissolve the resulting formazan precipitate. The optical density of the solution in each well was measured using the microplate reader at 570 nm wavelength.

Determination of apoptosis

Apoptosis was determined using Annexin-V Fluos Staining Kit (Roche, Germany). Following treatment, cells were harvested using accutase solution (Millipore, USA) and washed twice with phosphate-buffered saline (PBS) (Gibco, USA). The cells were later incubated with annexin-V fluorescein and propidium iodide (PI) for 10 min at room temperature. Cells were observed under the fluorescence microscope (Nikon, Japan) and a minimum of 10,000 events were collected and analyzed by flow cytometry, FACSCalibur (Becton Dickinson, USA). To determine whether ERα plays a role in 15d-PGJ$_2$-induced cell death, MCF-7 cells were pre-incubated with 100 nM ICI 182 780 (Popolo et al., 2011) for 1 h before treatment with 15d-PGJ$_2$ for 24 h. Following that, the cells were incubated with annexin-V and analysed by flow cytometry.

Western Blotting

Treated cells were collected using a cell scraper and washed twice with cold PBS. Cells were then lysed for 20 min in 250 µl of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.2 % sodium dodecyl sulphate (SDS), 1 mM phenylmethysulphonylfluoride, 2 µg/ml leupeptin, 2 µg/ml aprotinin and 1 mM sodium vanadate) and centrifuged at 12,000 rpm for 2 min. The supernatants were collected and the protein concentrations were determined using NanoDrop 2000 (Thermo Scientific, USA) at 280 nm absorbance. For analysis, 20 µg of protein were resolved on 10 % SDS-polyacrylamide gel and then transferred to a polyvinylidene diflouride membrane (Bio-Rad, USA) by semi-dry transfer. The membrane was blocked with 5 % skimmed milk at room temperature for 2 h and washed with Tris- buffered saline (TBS)-Tween 20 followed by overnight incubation with the primary antibody: ERα (Santa Cruz Biotechnology, USA), ERβ and β-actin (Abcam, USA). The membrane was washed again with TBS-Tween 20 and incubated with the secondary polyclonal antibody, goat anti-rabbit IgG-HBL (Abcam, UK) for 1 h. Protein bands were detected using ECL™ Western Blotting Analyses System (GE Healthcare, USA) and visualized using an image analyzer (DKSH, USA). The density of the bands was analyzed using the ImageJ 1.46 software [http://imagej.nih.gov/ij/] and the values were normalized to the β-actin band density.

Intracellular Ca^{2+} staining assay

After the cells were treated for 24 h, the culture medium was replaced with the new medium containing 5 µM Fluo-4 AM (Life Technologies, USA) and the cells were incubated at 37°C for 1 h. This was followed by washing with PBS and 30 min incubation to allow complete de-esterification of intracellular acetomethyl (AM) esters for fluorescence microscopy analysis.

Caspase activation

Treated cells were harvested using trypsin and washed with PBS. The cells were then re-suspended in 300 µl serum-free medium and incubated with 10 µl caspase-FLICA® of interest (8, 9 and 3/7) (ImmunoChemistry Technologies, USA) for 2h at 37°C in a 5 % CO_2. Following washing, the cells were analyzed by flow cytometry. Fluorescein (caspase- FLICA®) was measured on FL-1 channel with 10,000 collected events.

Statistical analysis

Data were obtained from three independent experiments. The values were expressed as mean ± standard deviation (SD). Statistical evaluation was performed using the Student t- test analysed using GraphPad Prism 5.01 (USA).

Results

Growth inhibitory effects of 15d-PGJ$_2$

15d-PGJ$_2$ caused dose-dependent cell death of MCF-7 and MDA-MB-231 cells with maximum cell death observed at 25 μM concentration (Figures 1A and 1B). The EC$_{50}$ values obtained ranged from 18 μM at 6 h to 5 μM at 72 h for MCF-7 cells, and from 11 μM at 6 h to 6 μM at 72 h for MDA-MB-231 cells. Constant EC$_{50}$ values obtained were 15 μM and 10 μM, respectively (Figures 1C and 1D). At these concentrations, 15d-PGJ$_2$ also significantly inhibited proliferation of both cell lines to 54 % for MCF-7 and 60 % for MDA-MB-231 (Figure 2).

Induction of apoptosis by 15d-PGJ$_2$

Figures 3A and 3B show typical morphological features of apoptosis of MCF-7 and MDA-MB-231 cells such as cell shrinkage and fragmentation into membrane-bound apoptotic bodies, after 24 h treatment with 15 μM and 10 μM 15d-PGJ$_2$, respectively. Apoptosis is also demonstrated by the intense green fluorescence of cells stained with annexin-V fluorescent antibody compared to the controls.

Flow cytometry analysis was further conducted to measure early and late apoptosis by 15d-PGJ$_2$. Treatment of MCF-7 cells with 15d-PGJ$_2$ caused 46 % total apoptosis compared to 10 % in the DMSO controls (p<0.01), with 40.5 % cells in late apoptosis and only 5.5 % in early apoptosis (Figure 3C). Similarly, 10 μM 15d-PGJ$_2$, caused 49.5 % total apoptosis of MDA-MB-231 cells compared
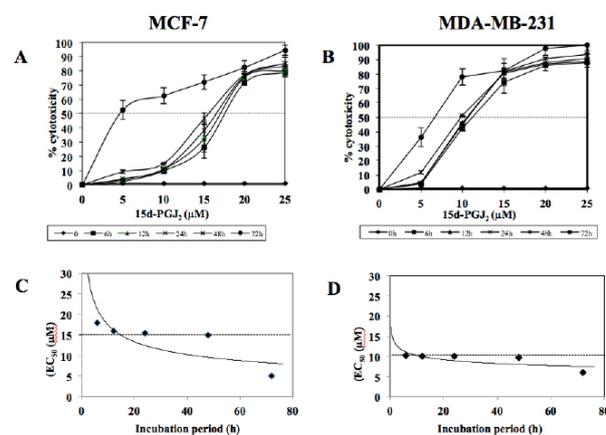

Figure 1. Dose Response Cytotoxic Effect of 15d-PGJ2 on (A) MCF-7 and (B) MDA-MB- 231 Cells and the EC$_{50}$ Values Against Each Time Point for (C) MCF-7 and (D) MDA-MB- 231 Cells Treated with 15d-PGJ2 (5-25μM) or DMSO (Control) for up to 72 h. Data are expressed as mean + SD of three independent experiments. *p< 0.05, significantly different from control cells
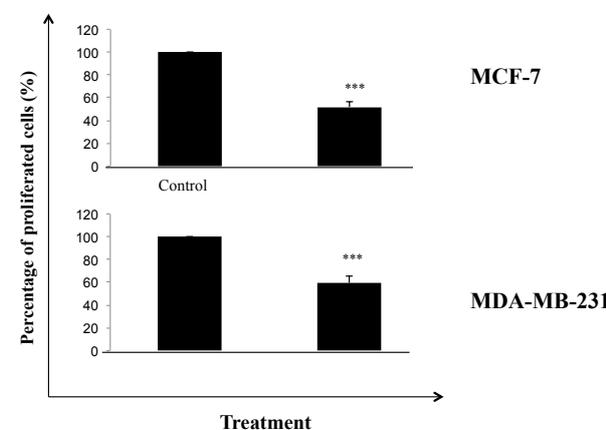

Figure 2. Effects of 15d-PGJ2 on Proliferation of (A) MCF-7 and (B) MDA-MB-231 Cells Treated with 15d-PGJ2 for 72 h. Data are expressed as mean ± SD from three independent experiments. **p < 0.01 and ***p<0.001 significantly different from control
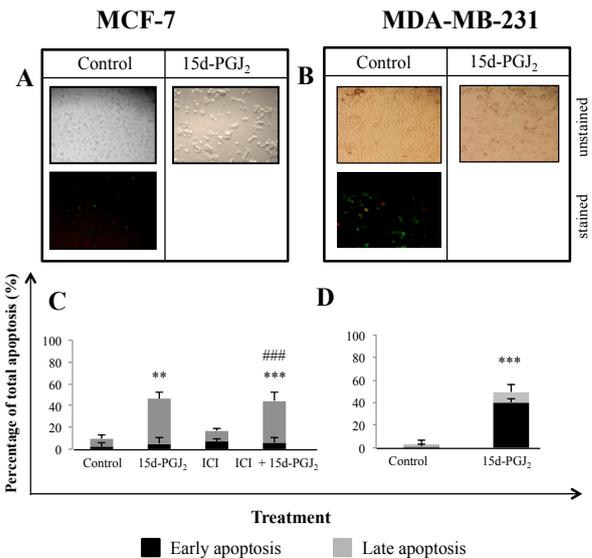

Figure 3. 15d-PGJ2-Induced Apoptosis in MCF-7 and MDA-MB-231 Cells with or without Exposure to ICI 182 780, Examined by Fluorescence Microscopy (A and B) and Quantified by Flow Cytometry (C and D). Data are expressed as mean ± SD from three independent experiments. **p < 0.01 and ***p < 0.001 significantly different from control and ### p < 0.001 significantly different from ICI 182 780
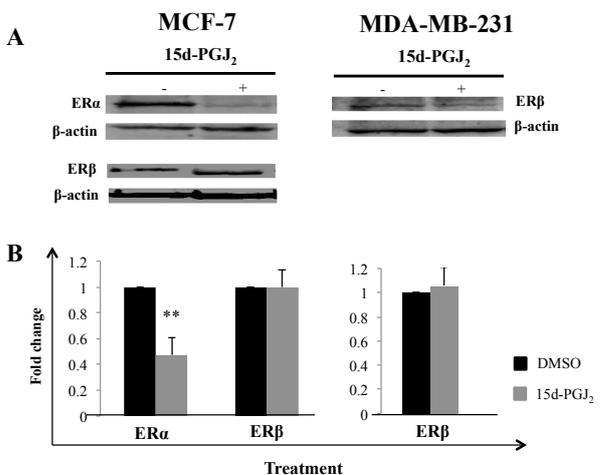

Figure 4. Expression of ERα and ERβ in MCF-7 and MDA-MB- 231 Cells Treated with 15d-PGJ2 (A) and Quantified Using Imagej Software (B). Data are expressed as mean ± SD from three independent experiments. **p<0.01 significantly different from control
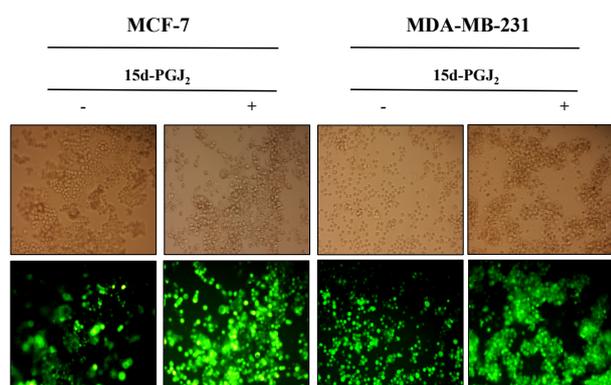
Figure 5. Intracellular Ca²⁺ Staining of MCF-7 and MDA-MB-231 Cells Treated with 15d- PGJ2. Images were captured using NIS elements BR version 2.20 at 200X magnification
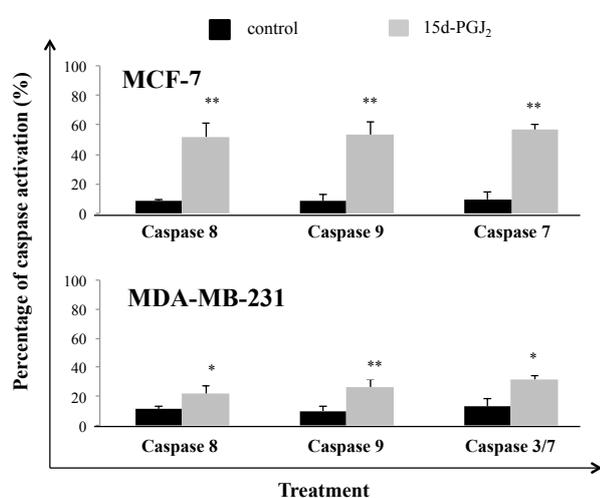

Figure 6. Caspase Activities in (A) MCF-7 and (B) MDA-MB-231 Cells Treated with 15d- PGJ2. Data shown are the mean ± SD of three independent experiments. *p < 0.05 and **p < 0.01 significantly different from control

to 3.6 % in controls (p<0.001). However, the percentage of cells in late apoptosis (9.2 %) is lower than that in early apoptosis (40.3 %, Figure 3D).

Role of ERα and ERβ in 15d-PGJ₂-induced apoptosis

In order to determine the involvement of ER signaling in 15d-PGJ₂-induced apoptosis of the ERα⁺ breast cancer cells, MCF-7 cells were pre-treated with ERα inhibitor, ICI 182 780, prior to 15d-PGJ₂ exposure. The percentage of total apoptosis in 15d-PGJ₂-treated cells pre-incubated with ICI 182 780 is 44.6 %, which is similar to the effect of 15d-PGJ₂ alone (Figure 3C), but is significantly higher (p<0.001) than the effect of ICI 182 780 alone (17.2 %). The results indicate that blocking the receptor ERα does not affect the apoptotic activity of 15d-PGJ₂. In addition, Western blotting analysis revealed that ERα expression was significantly reduced in MCF-7 cells by 15d-PGJ₂ (Figure 4). The expression of ERβ on the other hand, remained similar to control in both MCF-7 and MDA-MB-231 cells after treatment with 15d-PGJ₂.

Involvement of intracellular Ca²⁺ in 15d-PGJ₂-induced apoptosis

Increased intracellular Ca²⁺ has been shown to contribute to apoptosis (Orrenius et al., 2003). In the current study, increase in intracellular Ca²⁺ staining (green fluorescence) was observed in both MCF-7 and MDA-MB-231 cells compared to controls (Figure 5) following 24 h treatment with 15d-PGJ₂, suggesting the possible significance of intracellular Ca²⁺ concentration in 15d-PGJ₂ apoptotic action.

Activation of caspase-8, 9 and 3/7 activities by 15d-PGJ₂

Activation of caspase-8, 9 and 3/7 was determined using flow cytometry. In MCF-7 cells, 15d-PGJ₂ strongly activated the activity of caspase-8 (51.9 %), 9 (53.2 %) and 3/7 (56.6%) compared to control (DMSO 0.1%) (p<0.01, Figure 6A). Similarly, in MDA-MB-231 cells treated with 15d-PGJ₂, significant activation of caspase-8 (22.1%), 9 (26.5%) and 3/7 (31.9%) is also observed compared to control (p<0.05-0.01, Figure 6B).

Discussion

The PPARγ ligand, 15d-PGJ₂, significantly induced apoptotic cell death in both MCF-7 and MDA-MB-231. Although the levels of apoptosis were similar, significantly higher percentage of late apoptotic cells was noted for MCF-7 compared to MDA-MB-231. This is supported by the higher caspase activities observed in MCF-7 cells. In ERα⁺ breast cancer cells such as the MCF-7, disruption of ERα signaling pathway by 15d-PGJ₂ has been demonstrated through blocking or degradation of the ERα expression (Kim et al., 2007; Lecomte et al., 2008). In support, we showed that the protein expression of ERα was reduced in MCF-7 cells following treatment with 15 μM 15d-PGJ₂. We further blocked the ERα activity using the pure anti-estrogen, ICI 182 780 (also known as Fulvestrant) that is used for breast cancer treatment (Chen et al., 2014). ICI 182 780 acts as an inhibitor of ERα by downregulating this receptor and has no agonist effect. It can disrupt both the activation function sites AF-1 and AF-2 on ERα and accelerates degradation of ER protein due to the instability of ICI 182 780-ER complex (McKeage et al. 2004; Kocanova et al. 2010). We showed that the pre-treatment of ICI 182 780 failed to reduce the percentage of apoptotic cells further in 15d-PGJ₂ treatment of MCF-7 cells indicating that ERα does not influence the cell death activity of 15d-PGJ₂. The reduced ERα expression by 15d-PGJ₂ is in line with the reported proteasome-dependent degradation of ERα induced by 15d-PGJ₂ (Kim et al., 2007; Lecomte et al., 2008). This additionally implies that this PPARγ ligand could also suppress ERα-mediated proliferation that may contribute to the apoptotic effect observed in MCF-7 cells, but this needs to be confirmed in future studies. MDA-MB-231 cells are deficient of ERα hence blocking of receptor activity with ICI 182 780 was not performed. Next, we examined the role of ERβ in 15d-PGJ₂ apoptosis mechanism. Western blotting analysis showed that 15d-PGJ₂ did not modulate the expression of ERβ in either breast cancer cell lines, further confirming

the hormone receptor-independent nature of 15d-PGJ$_2$'s activity in breast cancer cells.

Caspases are intracellular proteases that play important roles during apoptosis. Reports on the involvement of caspases in the action of 15d-PGJ$_2$ are contradictory where both caspase-dependent (Shin et al., 2009) and caspase-independent (Cho et al., 2006) apoptotic cell death have been suggested. The discrepancy could probably be due to the use of different types of cancer cells. As for breast cancer, it has been reported that 15d-PGJ$_2$-induced apoptosis via caspase-dependent pathway in MDA-MB-231 cells but not in MCF-7 cells (Ciucci et al., 2006). On the contrary, we observed that activation of initiator caspase 8 and 9 as well as effector caspase 3/7 by 15d-PGJ$_2$ occurs in both MDA-MB-231 and MCF-7 cells. Although MCF-7 cells do not express caspase 3 due to functional 47-bp deletion in the exon 3 of CASP 3 gene, apoptosis can still occur by activating caspase 7 (Zoli et al., 2005; Twiddy et al., 2006). Activation of caspase 8 and 9 occurs through extrinsic and intrinsic pathways. Caspase 8 represents the extrinsic pathway that is initiated by the binding of death receptors (Fas and tumor necrosis receptor-1) to their ligands. Stimulation of death receptors leads to the formation of death-inducing signaling complex (Fas-associated via Death Domain [FADD] and procaspase 8) that activates caspase 8. Meanwhile, the mitochondrial pathway or intrinsic pathway involves the formation of apoptosome (association of cytochrome c, apaf-1 and procaspase 9) by internal stimuli such as DNA damage and oxidative stress. Activation of caspase 8 and 9 are important for the activation of effector caspase 3 and 7 (Parrish et al., 2013). Therefore based on the current study, 15d-PGJ$_2$ is capable of stimulating both the extrinsic and intrinsic pathways of apoptosis in breast cancer cells

Pro-apoptotic members of Bcl-2 family could increase the permeability of mitochondria to Ca^{2+} entry (Dejeana et al., 2006). The Bcl-2 causes the mitochondria to become stressed resulting in the formation of permeability transition pores (PTP) of mitochondria that is followed by cytochrome c release (Pacher and Hajnoczky, 2001; Decuypere et al., 2011). Calcium channels such as inositol triphosphate receptor (IP3R) are important to maintain the level of Ca^{2+} in the endoplasmic reticulum (Rizzuto et al., 2003; Monteith et al., 2007; Wen et al., 2016). However, increased basal Ca^{2+} level during apoptosis has led to the release of more Ca^{2+} to the cytosol through IP3R channel (Rao et al., 2004; Wen et al., 2016) resulting in elevated cytosolic Ca^{2+} level which altered the mitochondrial membrane potential and hence, increased Ca^{2+} entry into the mitochondria (Orrenius et al., 2003; Wen et al., 2016). We previously reported that apoptosis induction by 15d-PGJ2 was associated with perturbation of the mitochondrial membrane potential in both MCF-7 and MDA-MB- 231 cells (Yaacob et al., 2013). Herein, increased intracellular Ca^{2+} staining was observed in MCF-7 and MDA-MB-231 cells after 15d-PGJ2 treatment. This also supports the 15d- PGJ2-induced activation of caspase 9 observed in the current study that could be a result of Ca^{2+}-induced mitochondrial swelling and cytochrome c release (Landar et al., 2006). A similar observation was reported by Yu et al. (2008) where DE-71 induced

apoptosis via increased intracellular Ca^{2+} levels in human neuroblastoma cells which led to Bax translocation to the mitochondria, release of cytochorome c and activation of caspase activity. In addition, the Ca^{2+} level is also increased when caspase 8 is dissociated from caspase recruitment domain, CARD (Jo et al., 2004).

In conclusion, the action of 15d-PGJ$_2$ seems to be pleiotropic. Pro-apoptotic effects of 15d-PGJ$_2$ are the results of perturbation of mitochondrial membrane potential and other mechanisms involving elevated intracellular Ca^{2+} and caspase-dependent intrinsic and extrinsic pathways. In addition, our findings suggest that disruption of ERα-signaling pathway but not ERβ could also contribute to the apoptotic ability of 15d-PGJ2. Modulation of the intracellular Ca^{2+} level may also have implications on cancer cell migration. However, further studies are needed to maximize the beneficial effects of 15d-PGJ$_2$ where it will provide an insight to the role of 15d-PGJ$_2$ as a potential anticancer agent for treatment of breast cancer.

Acknowledgements

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100.0
6.3
75.0
56.3
50.0
25.0
31.3
0

Newly diagnosed without treatment