RESEARCH ARTICLE

Influence of 17β-Estradiol on 15-Deoxy-$\Delta^{12,14}$ Prostaglandin J_2 -Induced Apoptosis in MCF-7 and MDA-MB-231 Cells

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Abstract

The nuclear receptor, peroxisome proliferator-activated receptor gamma (PPARγ), is expressed in various cancer cells including breast, prostate, colorectal and cervical examples. An endogenous ligand of PPARγ, 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2 (PGJ2), is emerging as a potent anticancer agent but the exact mechanism has not been fully elucidated, especially in breast cancer. The present study compared the anticancer effects of PGJ2 on estrogen receptor alpha (ERα)-positive (MCF-7) and ERα-negative (MDA-MB-231) human breast cancer cells. Based on the reported signalling cross-talk between PPARγ and ERα, the effect of the ERα ligand, 17β-estradiol (E2) on the anticancer activities of PGJ2 in both types of cells was also explored. Here we report that PGJ2 inhibited proliferation of both MCF-7 and MDA-MB-231 cells by inducing apoptotic cell death with active involvement of mitochondria. The presence of E2 potentiated PGJ2-induced apoptosis in MCF-7, but not in MDA-MB-231 cells. The PPARγ antagonist, GW9662, failed to block PGJ2-induced activities but potentiated its effects in MCF-7 cells, instead. Interestingly, GW9662 also proved capable of inducing apoptotic cell death. It can be concluded that E2 enhances PPARγ-independent anticancer effects of PGJ2 in the presence of its receptor.

Keywords: 15 deoxy-prostaglandin J_2 - 17β-estradiol - apoptosis - MCF-7 - MDA-MB-231 - GW9662

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Introduction

Peroxisome proliferator-activated receptor γ (PPARγ) is over-expressed in many different tumour types and drugs that activate PPARγ are widely studied for their ability to inhibit tumour cell growth. PPARγ ligands include the naturally occurring 15-Deoxy-$\Delta^{12,14}$ prostaglandin J_2 (PGJ2), and synthetic oral antidiabetic drugs, thiazolidinediones, and non-steroidal anti inflammatory drugs. PGJ2 is the terminal derivative of prostaglandin J_2 reported to have potent anticancer effects in human cancers of various origins (Bailey and Hla, 1999; Nikitakis et al., 2002; Kamagata et al., 2007; Ferreira-Silva et al., 2008; Apostoli et al., 2013; Chang and Hu, 2013; Kumar et al., 2013). On the other hand, the proliferative role of PGJ2 in mammary and colon carcinogenesis has also been reported (Choi et al., 2008; Kim et al., 2008). Importantly, several reports indicate that PGJ2 effects occur either dependently or independently of PPARγ (Clay et al., 2000; 2002; Ray et al., 2006; Ferreira-Silva et al., 2008; Chbicheb et al., 2011).

PPARγ binds to specific recognition sites on DNA, known as the peroxisome proliferator response elements (PPREs), as a heterodimer complex with retinoid X receptor (Kliewer et al., 1992; Mukherjee et al., 1997) to modulate target genes and interaction between PPARγ and the estrogen receptor, ERα, has been reported in breast cancer cells (Wang and Kilgore, 2002; Bonofiglio et al., 2005; Lee et al., 2009). ERα lowered both basal and stimulated PPARγ-mediated reporter activity (Wang and Kilgore, 2002) and repressed the transactivation of PPRE in cancer cell lines (Bonofiglio et al., 2005). In the present study, the influence of E2 on the cell death effect of PGJ2 on ERα-positive (MCF-7) and ERα-negative (MDA-MB-231) breast cancer cell lines was investigated. Although PGJ2 induced apoptosis and altered the mitochondrial membrane potential in both cell lines, E2 differentially affect these activities in both cells. Direct involvement of PPARγ in the cell death mechanism of these breast cancer cell lines was also assessed using a potent, irreversible PPARγ specific antagonist, 2-chloro-5-nitrobenzanilide (GW9662), in the presence or absence of PPARγ and ERα agonists.

Materials and Methods

Cell lines and reagents

Human breast cancer cell lines, MCF-7 and MDA-MB-231, were maintained in RPMI and DMEM, respectively, supplemented with 10% fetal bovine serum (Hyclone, USA), penicillin (100 units/ml) and streptomycin (100 mg/ml) (Gibco, USA). PGJ2 (Cayman

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Chemicals, USA) was dissolved in dimethyl sulphoxide (Sigma, USA) at a concentration of 100μM. E2 (Sigma, USA) was dissolved in ethanol at a concentration of 1μM/ml.

Treatment of cells

MCF-7 and MDA-MB-231 cells were seeded in T-25 cm2 flasks or in chamber slides (for apoptosis assay) at a density of 2×10^5 and 1×10^6 cells/ml, respectively until about 70% confluent. MCF-7 and MDA-MB-231 cells were treated with 15μM and 10μM PGJ2, respectively, 10nM E2 or their combination. For PPARγ blocking, cells were pre-treated with 10μM GW9662 for 1h prior to treatment with the agonists. The solvent, dimethyl sulphoxide (less than 0.15%) or ethanol (less than 1%) was used for untreated control cells. Cells were then harvested at different incubation periods and subjected to subsequent experiments.

Annexin V-FLUOS assay

Apoptosis was analyzed using the Annexin V FLUOS Staining Kit (Roche, Germany) according to the manufacturer's instructions. Briefly, treated and untreated cells were harvested by trypsinization (with 0.025% trypsin) at 6, 24, 48 and 72 h. The cells were pelleted by centrifugation at 1000 rpm for 5 min, and washed with phosphate-buffered saline. The cell pellet was then resuspended in 100μl Annexin V FLUOS labeling solution (20μl Annexin V-FLUOS labeling reagent and 20μl Propidium iodide (PI) solution in 1ml incubation buffer) for 10-15 min at room temperature. A minimum of 10,000 events were collected and analyzed using the flow cytometer (FACS Calibur, Becton-Dickinson, USA) with Cellquest software (Becton-Dickinson, USA).

Mitochondrial membrane potential

Variation in the mitochondrial transmembrane potential ($\Delta\Psi m$) was determined using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) fluorescence staining. Treated and untreated cells were harvested by trypsinization at 6, 24 and 48 h and incubated with JC-1 dye dissolved in serum-free media at a working concentration of 5 μM, for 30 min at 37°C, according to the manufacturer's instructions. The cells were then washed and resuspended in phosphate-buffered saline for analysis by flow cytometry.

Statistical analysis

The experiments were repeated three times and the significant differences in the mean of treated and untreated cells were calculated using Mann-Whitney U test using SPSS 12.0 software.

Results

Induction of apoptosis in MCF-7 and MDA-MB-231 cells by PGJ2 alone and in combination with E2

Apoptosis was determined using fluorescence conjugated Annexin V antibody that detects and binds to phophatidylserine (PS) exposed onto the surface of cells at their early stages of apoptosis, and PI that enters the permeable plasma membrane of cells in their late stages of apoptosis or cells undergoing necrosis. Analysis of cells stained with Annexin V antibody and PI flow cytometry showed that PGJ2 alone or in combination with E2 significantly induced higher levels of apoptosis in MCF-7 cells at all incubation periods compared with untreated controls ($p<0.05$), killing more than 80% cells at 72h (Figure 1). Most of these apoptotic cells were found to be in the late stage of apoptosis. Apoptosis in MCF-7 cells treated with the combination of PGJ2 and E2 remained significantly higher (almost 20%) than cells treated with PGJ2 alone ($p<0.05$). Cells treated with E2 alone however showed similar levels of apoptosis as the untreated cells, except at 72 h incubation where more apoptotic cells were observed with E2 treatment ($p<0.05$). However, apoptotic levels of cells treated with E2 remained significantly lower than those treated with PGJ2 and PGJ2-E2 combination. Low levels of necrosis were observed throughout the experiment (results not shown).

For MDA-MB-231 cells, significant cell death was evident 24h following the combination treatment (40-50%) and this was further enhanced at 48 and 72h (>80%), mainly due to increased late stage apoptosis (Figure 2). However, unlike MCF-7 cells, addition of E2 to PGJ2-treated MDA-MB-231 cells did not show any significant difference in apoptosis compared to cells treated with PGJ2 alone. Treatment with E2 alone did not

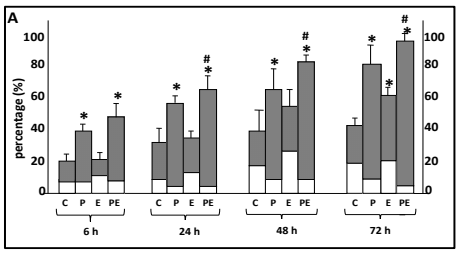

Figure 1. Induction of Apoptosis in MCF-7 Cells Treated with PGJ2, E2 and PGJ2+E2 Combination. Cells were treated with 15μM PGJ2, 10nM E2 or PGJ2+E2 combination for 6, 24, 48 and 72h. The cells were incubated with Annexin-V antibody and propidium iodide dye and analyzed by flow cytometry. Each bar in represents the mean±SD. of three independent experiments. *$p<0.05$ significantly different from untreated cells. #$p<0.05$ significantly different from PGJ2+E2 treated cells

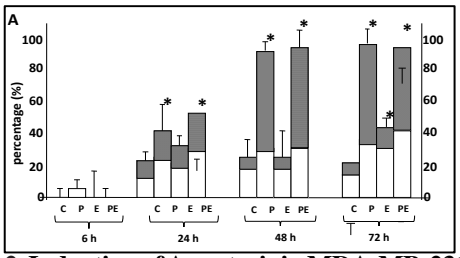

Figure 2. Induction of Apoptosis in MDA-MB-231 Cells Treated with PGJ2, E2 and PGJ2+E2 Combination. Cells were treated with 10 μM PGJ2, 10 nM E2 or PGJ2+E2 combination for 6, 24, 48 and 72 h. The cells were incubated with Annexin-V antibody and propidium iodide dye and analyzed by flow cytometry. Each bar in represents the mean±SD. of three independent experiments. *$p<0.05$ significantly different from untreated cells. #$p<0.05$ significantly different from PGJ2+E2 treated cells

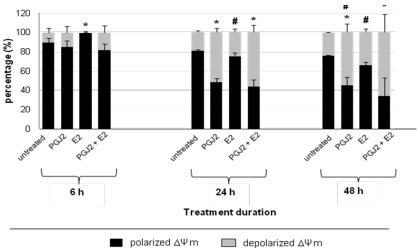

Figure 3. Analysis of Alterations in Mitochondrial Membrane Potential in MCF-7 Cells Treated with PGJ2, E2 and Their Combination. MCF-7 cells were treated with 15 μM PGJ2 and 10 nM E2 alone or in combination for 6, 24 and 48 h. Cells were then stained with JC-1 dye for flow cytometric analysis. Each bar represents the mean±SD. of three independent experiments. *p<0.05 significantly different from untreated control cells. #p<0.05 significantly different from PGJ2+E2-treated cells

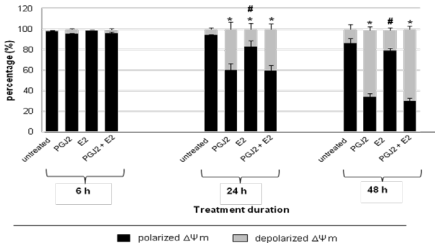

Figure 4. Analysis of Alterations in Mitochondrial Membrane Potential in MDA-MB-231 Cells Treated with PGJ2, E2 and Their Combination. MDA-MB-231 cells were treated with 10μM PGJ2 and 10nM E2 alone or in combination for 6, 24 and 48h. Cells were then stained with JC-1 dye for flow cytometric analysis. Each bar represents the mean±SD. of three independent experiments. *p<0.05 significantly different from untreated control cells. #p<0.05 significantly different from PGJ2+E2-treated cells

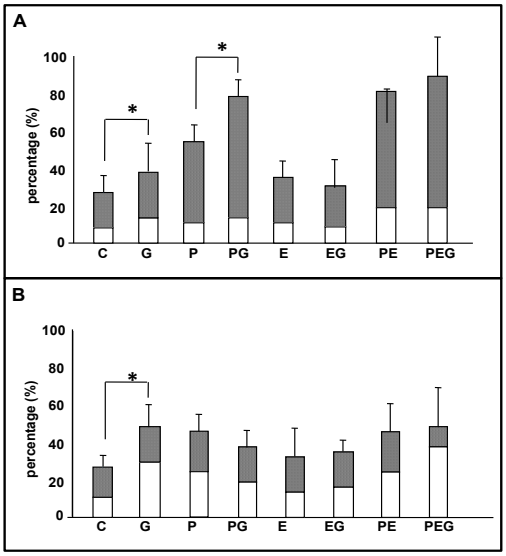

Figure 5. Effect of GW9662 on PGJ2-Induced Apoptosis in (A) MCF-7 and (B) MDA-MB-231 Cells. Cells were treated with various combinations of PGJ2, E2 and GW9662 for 24 h. The cells were stained with Annexin-V antibody and propidium iodide and analyzed by flow cytometry. Each bar represents the mean +SD. of three independent experiments. *p< 0.05 significantly different from corresponding group of cells not treated with GW9662

show any significant difference in cell death compared to untreated cells, except at 72h, but remained significantly lower compared to cells treated with PGJ2 alone and in combination with E2.

Changes in the mitochondrial membrane potential in MCF-7 and MDA-MB-231 cells treated with PGJ2 and E2 alone and in combination

JC-1 dye is a lipophilic fluorochrome that penetrates the plasma membrane of cells as monomers (green fluorescence), while entering the mitochondria as aggregates (red fluorescence). The membrane of healthy and normal mitochondria is polarised and thus, JC-1 is rapidly taken up by such mitochondria, whereas, unhealthy mitochondria with compromised membrane, are impermeable to the JC-1 dye which then concentrates in the cytoplasm. Treated and untreated MCF-7 and MDA-MB-231 cells labeled with JC-1 dye were analysed by flow cytometry. Significant ($p<0.05$) decrease in the $\Delta\Psi m$ (an increase in mitochondrial depolarization and a decrease in mitochondrial polarization) was evident after 24 and 48 h treatment (Figures 3 and 4). Both cell lines treated with E2 alone did not show any significant differences from untreated cells, except for a small decrease in the $\Delta\Psi m$ in MDA-MB-231 cells at 24h of incubation. There was no significant difference in $\Delta\Psi m$ between cells treated with PGJ2 alone and PGJ2 in combination with E2 at all time points in MDA-MB-231 cells. In contrast, MCF-7 cells treated with the combination of both ligands showed significantly higher ($p<0.05$) levels of mitochondrial depolarization than cells treated with PGJ2 alone at 48h.

Effect of GW9662 on PGJ2 induced cell death of MCF-7 and MDA-MB-231 cells

The role of PPARγ in the modulation of apoptosis in breast cancer cells by PGJ2 in the presence or absence of E2 was examined following complete inhibition of PPARγ activity using a potent PPARγ antagonist, GW9662, which binds irreversibly to the ligand-binding domain of the receptor (Leesnitzer et al., 2002; Seargent et al., 2004). Blocking of PPARγ did not affect apoptotic cell death activity induced by the combination of PGJ2 and E2 but resulted in 20% increase in the percentage of apoptosis induced by PGJ2 alone in MCF-7 (A) but not in MDA-MB-231 cells (Figure 5B). It is also noteworthy that GW9662 itself produced a 40-50% increase in apoptosis of both breast cancer cells compared to untreated controls.

Discussion

ERs act as transcriptional factors by binding to estrogen response elements (EREs) on target genes, facilitated by interaction with corepressors or coactivators (Gupta and Kuperwasser, 2006; Lee et al., 2013). PGJ2 has been reported to inhibit E2-mediated ERE transactivation in leiomyoma cells (Houston et al., 2003) and growth of MCF-7 cells partly via proteasome-dependent degradation of ERα (Qin et al., 2003). The inhibitory influence of PPARγ agonists on ERα expression is supported by other reports of negative interference of PPARγ and ERα on each others' transactivation via response element binding

(Keller et al., 1995; Wang and Kilgore, 2002; Bonofiglio et al., 2005; Kim et al., 2007; Jeong and Yoon, 2011). However, a previous study reported that inhibition of either E2-dependent or E2-independent ERα transcription by PGJ2 occurred independent of PPARγ (Kim et al., 2007). In the current study, the ERα-expressing MCF-7 cells and non-expressing MDA-MB-231 cells (Lee et al., 2005) were used as human breast cancer models to investigate cancer cell death activities of the PPARγ ligand, PGJ2, and the influence of the ERα agonist, E2, to influence PPARγ ligand-induced activities.

PGJ2 has been reported to inhibit proliferation of breast cancer cells either via apoptosis (Clay et al., 1999) or other non-apoptotic mechanisms (Qin et al., 2003). In the current study, PGJ2 was found to induce apoptosis in both ERα-positive MCF-7 and ERα-negative MDA-MB-231 breast cancer cells suggesting that the ER may not have any influence on PGJ2's ability to induce apoptosis in breast cancer cells. The induction of apoptosis occurred much earlier in MCF-7 cells (from 6h) compared to MDA-MB-231 cells (starting at 24h). In addition, mitochondrial membrane depolarization was induced by PGJ2 in both MCF-7 and MDA-MB-231 cells. The mitochondria play a critical role in apoptosis by releasing cytochrome c and other proteins that are essential for the execution of apoptosis. Our findings are in agreement with previous reports of PGJ2-induced mitochondrial dysfunction and ROS production in MCF-7 cells (Pignatelli et al., 2005; Kim et al., 2008). In order to determine whether PGJ2 apoptotic action is dependent on PPARγ activation, 10 μM of the irreversible PPARγ antagonist, GW9962, was used to completely block PPARγ active site (Seargent et al., 2004; Kourtidis et al., 2009). GW9962 is also suitable because it does not affect PPARγ-mediated transcription (Leesnitzer et al., 2002). The use of GW9662 in both MCF-7 and MDA-MB-231 cells failed to block PGJ2-induced apoptosis indicating that this event occurs independently of PPARγ activation. This is in agreement with the report of PPARγ-independent cytotoxicity of B cell lymphoma (Ray et al., 2006), Jurkat, HeLa and U937 cells (Ferreira-Silva et al., 2008; Theoleyre et al., 2010) by PGJ2. Thiazolidinedione, a synthetic PPARγ ligand, has also been found to produce receptor-independent antitumour effects (Wei et al., 2009). In addition, we observed that blocking PPARγ activation resulted in increased PGJ2-induced apoptosis, further supporting a receptor-independent action of this ligand. The potent apoptotic activity of PGJ2 on MCF-7 cells may be attributed to its ability to induce proteasomal degradation of ERα in these ER-positive cells, thus inhibiting cellular proliferation (Qin et al., 2003; Lecomte et al., 2008), via enhanced ubiquitination. PPARγ-induced intracellular degradation of the receptor has also been reported (Qin et al., 2003), in line with the reported negative interaction between PPARγ and ERα (Keller et al., 1995; Wang and Kilgore 2002; Bonofiglio et al., 2006).

We showed that the presence of E2 potentiated PGJ2-induced apoptosis in MCF-7 but not in MDA-MB-231 cells. Apoptosis of PGJ2+E2-treated MCF-7 cells remained about 10% higher than those treated with PGJ2 alone at all time points showing that E2 treatment has an additive effect on PGJ2-induced cell death activity. In fact E2 has previously been reported to induce apoptosis in hormone-dependent breast cancer cells (Song et al 2001; Gregoraszczuk and Ptak, 2011) either through the extrinsic death receptor pathway or via the intrinsic pathway of mitochondrial disruption and release of cytochrome C. Our study did in fact demonstrate that E2 alone caused apoptosis at 72h post-treatment although the apoptosis-potentiating effect of E2 on PGJ2 occurred earlier (at 24h), which may occur through the extrinsic death receptor pathway as previously suggested (Song et al., 2001).

Interestingly however, potentiation of PGJ2 response by E2 was not observed in MDA-MB-231 cells. Although PGJ2 was capable of apoptotic activity in the presence or absence of ERα, the promoting action by E2 seems to be influenced by the presence of the receptor. Our findings further showed that E2 potentiates PGJ2-induced mitochondrial membrane depolarisation in MCF-7 and not in MDA-MB-231 cells which further suggests for a role of ERα in the promotion of PGJ2-induced cell death mechanism by estradiol. PGJ2 itself is said to be capable of inhibiting both hormone-dependent and hormone-independent ERα transcriptional activity via covalent modification of cysteine residues within the vulnerable COOH-terminal zinc finger of ERα DNA binding domain (Kim et al., 2007) and could trigger proteasomal degradation of ERα in a PPARγ-independent manner (Lecomte et al., 2008).

The ability of GW9662 alone to cause apoptosis of both cells further supports the notion that PPARγ does not play a significant role in breast cancer cell death. In fact, direct antiproliferative effect of GW9662 has previously been reported in MCF-7, MDA-MB-231 and MDA-MB-468 cells (Sergeant et al., 2004; Malaviya and Sylvester, 2013). A higher concentration of the antagonist (20 μM) was also reported to cause extensive cell death (Kim et al., 2008). In addition, we found that the presence of the antagonist augmented the anticancer effect of PGJ2 in the ERα-positive cells which was not observed in the ERα-negative cells, further suggesting a negative regulatory activity between PPARγ and ERα perhaps via competition for co-regulatory proteins. The mechanism of how GW9662 is antiproliferative in MCF-7 cells is still unclear but interference on the inhibitory action of PGJ2 on ERE-dependent transactivation by E2 has been suggested (Kim et al., 2007).

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In conclusion, the mechanism of anticancer action of PGJ2 on breast cancer cells seems to be pleotropic but several data including the current findings provide evidence that the anticancer effects are the results of PPARγ-independent events. The apoptosis induced occurs via perturbation of mitochondrial membrane potential and is enhanced by estrogen in the presence of its receptor. Further studies using a panel of ER-positive and-negative breast cancer cell lines and other PPARγ ligands, and perhaps ER-specific chemotherapeutic drugs such as tamoxifen should be carried out to determine whether combination of such drugs could enhance apoptosis of breast cancer cells.

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