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EP4 mediates PGE₂ dependent cell survival through the PI3 Kinase/AKT pathway

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Abstract

The anti-apoptotic effect of PGE₂ was examined in Jurkat cells (human T-cell leukemia) by incubation with PGE₂ (5nM) prior to treatment with the cancer chemotherapeutic agent camptothecin. Apoptosis was evaluated by caspase-3 activity in cell extracts and flow cytometry of propidium iodide-labeled cells. Pre-incubation with PGE₂ reduced camptothecin-induced caspase activity by 30% and apoptosis by 35% respectively. Pharmacological data demonstrate that the EP4 receptor is responsible for mediating the protection from camptothecin-induced apoptosis. Pre-treatment of the cells with the EP4 antagonist (EP4A) prior to PGE₂ and camptothecin abolished the increased survival effect of PGE₂. Specific inhibition of the downstream of PI3 kinase or AKT/protein kinase but not protein kinase A prevents the observed increase in cell survival elicited by PGE₂. These findings have critical implications regarding the mechanism and potential application of PGE₂ receptor specific inhibition in cancer therapy.

Keywords

Prostaglandin; EP4 receptor; Apoptosis; Survival; PI3 Kinase

1. INTRODUCTION

Prostaglandin E₂ has wide-ranging effects on many tissues (1). One response that PGE₂ elicits in many different cell types is an increase in survival following stimuli that normally lead to apoptosis (2-4). Over expression or inhibition of cyclooxygenase (COX) and the resulting changes in PGE₂ levels, directly influence the sensitivity of cells to apoptotic stimuli (5). Prostaglandin dependent survival is an important cellular defense mechanism and also plays an important role in carcinogenesis(6). There is an abundance of evidence implicating PGE₂ functioning in tumor initiation and progression as well as being a mechanism of resistance to radiation or chemotherapy. The PGE₂ specific signaling events that contribute to neoplastic transformation in mammalian cells remain difficult to elucidate, although the regulation of apoptosis has been identified as a central mechanism underlying tumorigenesis in cancers over-expressing Cox-2 (5,7). PGE₂ exerts its biological effects by binding to four distinct G-coupled receptors, termed EP receptors (EP1-EP4) (8). While the EP1 receptor couples to G_iq and elevates intracellular calcium, EP3 has multiple splice variants which may couple through G_is,

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G β or G α_q (9). EP2 and EP4 couple to the G β s and stimulate cAMP production. The data available suggest that the PGE $_2$ dependent survival response is mediated by either or both EP2 and EP4 receptors. Hishino et. al. reported that specific agonists to EP2 and EP4 protected gastric mucosal cells from alcohol induced apoptosis (10). The protection mediated through either receptor was blocked by the protein kinase A inhibitor, H89, indicating that the survival signal is mediated by cAMP dependent Protein Kinase A. Protection of T24 bladder and T84 intestinal (11,12), epithelial cells by PGE $_2$ correlated with and was mimicked by elevation of cAMP, suggesting that EP2 and/or EP4 receptors mediate the protective response. We have previously reported that protection of the gut from γ -irradiation in mice was mediated by the EP2 receptor based upon decreased survival rates in EP2 α knockout mice (4). Tessner et. al. similarly concluded that PGE $_2$ protection from γ -irradiation in HCT-116 cells was mediated by EP2 although indirectly through transactivation of EGF receptors (13). Machwate et. al. however reported that PGE $_2$ dependent protection of PR-1 periosteal cells from apoptosis induced by low serum was reversed by an EP4 specific antagonist (14). Since EP4 can transduce the PGE $_2$ signal through either elevation of cAMP or activation of PI3 kinase, a detailed examination of how EP4 mediates PGE $_2$ -dependent protection is of interest. Jurkat cells provide an excellent model system in which to analyze the mechanisms underlying EP4 mediated PGE $_2$ survival response. The EP4 receptor was originally described by DeVries in Jurkat cells (15) which are reported to have limited expression of additional EP receptors (16),(17). The induction of apoptosis by the chemotherapeutic agent camptothecin has been well characterized in these cells. In the present study, we investigated the role of EP4 mediated signal transduction in the regulation of camptothecin-induced apoptosis.

2. METHODS AND MATERIALS

2.1. Materials

Prostaglandin E2, Prostaglandin E1-alcohol, sulprostone, and butaprost were obtained from Cayman Chemical. Camptothecin and H89 were from Sigma Chemical. EP4A was a gift from Kathleen Metters (Merck-Frosste). Stock solutions of all compounds were made in DMSO and diluted in media. Final DMSO concentration in treated wells and vehicle controls were matched and less than 0.5%. LY294002 and Wortmannin were obtained from Cell Signaling Technology. AKTi (1L-6-Hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate) 4-Cyano-3-methylisoquinoline, and cell permeable, Protein Kinase A Inhibitor 14-22 Amide were obtained from Calbiochem. Antibodies to prostaglandin E2 receptors (EP1-4, Catalog #101740; 101750; 101760 and 101775) and corresponding blocking peptides were from Cayman Chemical. Antibodies to pBad -Ser136 (sc-12969); AKT (sc-8312) and pAKT-Ser473 (sc-7985R) were from Santa Cruz.

2.2. Cell and Culture conditions

Jurkat cells (TIB-152), Clone E6-1, were obtained from the ATCC. Jurkat cell were maintained between a density of 0.15-1.5 $\times 10^6$ cells /ml in RPMI1640 media with 10% Fetal Bovine Serum supplemented with 2mM glutamine and antibiotics. For FACS or caspase analysis of apoptosis, prostaglandins were added 1hr prior to addition of camptothecin. Pathway specific inhibitors were added 30 minutes prior to prostaglandin addition. Inhibitors were added at a concentration between 2 and 10 times their reported inhibition constants.

2.3. FACS analysis

1.5 $\times 10^6$ cells in 2.5mls were aliquoted to 6 well plates and were treated for 12 hours with 25uM camptothecin. Cells were washed with PBS, fixed with 70% ethanol at 4 $^{\circ}$ C for 1 hour, washed 2 times with PBS and then resuspended in PBS containing propidium iodide, 50ug/ml and RNase A, 0.25mg/ml.

2.4. Caspase assays

Cells were diluted to a density of 5×10^5 cells/ml in fresh media and 2.5×10^4 cells aliquoted to a 96 well opaque plate. Caspase activity was assayed 6 hours after camptothecin addition by measuring fluorescence at 481/521 nm of Promega's Apo-One Caspase 3/7 reagent (Z-DEVD-R-110) using Synergy HT fluorometer. Camptothecin at 25 μ M has been previously shown to induce apoptosis within 6 hrs in Jurkat cells (18).

2.5. Western Blotting

Western analysis was performed on cells harvested in 50 μ L RIPA buffer containing 1mM NaF, 1mM NaVO₄ and 0.1mM PMSF. Twelve micrograms of soluble protein was loaded per lane. Proteins were transferred to nitrocellulose membranes, blocked with 2% dried milk/0.5% Tween-20 and incubated with primary antibodies. Primary antibodies to the EP receptors used at a dilution of 1:1000 and incubated overnight at 4°C. Blocking peptides to EP3 and EP4 were preincubated with diluted antibody at a concentration of 0.5 μ g/ml. The primary antibody to pBAD 136 was used at a dilution of 1:500. The primary antibody to AKT and pAKT were used at a dilution of 1:1000. Antibodies to phosphorylated proteins were incubated for 1 hour at room temperature with NaF present.

2.6. Real-time Quantitative PCR

Total RNA samples were extracted from cells with Trizol. To prepare cDNAs, these samples were reverse transcribed with SuperScript III, T7 Oligo (dT) primer, and Random Primers. Reaction conditions were: 25°C for 10 min., 50°C for 50 min., 70°C for 15 min. The cDNA samples were then amplified using iQ SYBR Supermix at 95°C for 3 min., then 95°C for 15 sec. and 62°C for 15 sec. for a total of 40 cycles. All PCR reactions were performed in triplicate in an iCycler iQ real-time PCR detection system. Relative mRNA levels were assessed by standardization β -actin). The EP real time primers were confirmed using HCT116 cells (not shown). Results were expressed as an n-fold difference in gene expression. Sequences of the PCR primers pairs used were: huEP1 (forward) 5'-GTG GAG ATG GTG GGC CAG-3'; huEP1 (reverse) 5'-GCC ACC AAC ACC AGC ATT G-3'; huEP2 (forward) 5'-GAC CAC CTC ATT CTC CTG GCT A-3'; huEP2 (reverse) 5'-TCC TTT CGG GAA GAG GTT TCA-3'; huEP3 (forward) 5'-GAT CAT GTG CGT GCT GTC G-3'; huEP3 (reverse) 5'-CCG TGT GTG TCT TGC AGT GC-3'; huEP4 (forward) 5'-CAT CTG CTC CAT CCC GCT-3'; huEP4 (reverse) 5'-GGA TGG CCT GCA AAT CTG G-3'.

3. RESULTS

3.1. Prostaglandins are protective

Camptothecin treatment of Jurkat cells result in an induction of caspase activity (Figure 1A) leading to apoptosis (Figure 1B). Caspase activity was markedly increased when assayed 6 hours after camptothecin addition to the media. When cells were harvested 12 hours after camptothecin treatment and stained with propidium iodide for FACS analysis, 40% of the cells contain subdiploid DNA content, indicative of apoptosis. Treatment of the cells with 5nM PGE₂ prior to the addition of camptothecin reduced the level of caspase activity by 30% and diminished apoptosis by 35%, respectively. The dose response curve for PGE₂ (Figure 1C) was maximal between 2 and 10 nM, similar to published receptor binding data for the EP receptors (19-21).

3.2. EP receptor expression in Jurkat cells

Characterization of EP receptor expression in Jurkat cells by real-time RT PCR (Figure 2A) and immunoblotting (Figure 2B) indicate that the cells express EP3 and EP4 prostaglandin receptors. By real-time PCR analysis, the EP4 mRNA level is approximately 4 times greater

than the level of EP3, although quantitation of Western blots indicate that EP3 protein levels are twice as great as EP4. Pre-incubation of the receptor antibodies with their respective blocking peptide confirmed the specificity of receptors (Figure 2C). Real-time PCR analysis indicated no expression of either EP1 or EP2 mRNA in these cells.

3.3. Protection is mediated by EP4

Pharmacological characterization of the survival effects of PGE₂, as measured by the attenuation of camptothecin-induced caspase activity, indicates that EP4 is responsible for mediating the protection of Jurkat cells (Figure 3). Prostaglandin E1-alcohol, an EP3/4 agonist, was as effective as PGE₂ in protecting the cells, but sulprostone, an EP1/3 agonist elicited no protection. The dose response curve for prostaglandin E-1 alcohol indicated that when PGE1-alcohol was added at concentrations above 1 μ M to Jurkat cells prior to camptothecin, caspase activity was reduced by 30 percent. The EC50 for PGE1-alcohol was approximately 200nM. Butaprost, an exclusively EP2 agonist had no protective effect. Pre-treatment of the cells with the EP4 antagonist EP4A(14) prior to PGE₂ (or Prostaglandin E1-alcohol, data not shown) prevented the increased survival response (Figure 4a). PGE₂ dose response curves in the presence of increasing concentrations of EP4A (Figure 4b) resulted in comparable decrease in caspase activity but at corresponding higher concentrations of PGE₂. Schilds analysis of the inhibition by EP4A indicated that the compound displayed an IC50 of $\sim$ 20nM.

3.4. EP4 dependent protection is mediated by PI3 kinase pathway

Downstream signaling by the G α s coupled EP4 receptor may be through either the Protein Kinase A or PI3 kinase pathways. Pretreatment of the cells with a protein kinase A inhibitor, H89, shown in Figure 5 (or 4-Cyano-3-methylisoquinoline or the cell permeable, myristolated peptide PKA Inhibitor 14-22 Amide, not shown) failed to block the pro-survival effect of PGE₂. Addition of PI3 kinase inhibitors, Ly294002 or Wortmannin, however, completely blocked the PGE₂ response (Figure 5). Specific inhibition of the downstream AKT/protein kinase B by the AKT inhibitor (AKTi) also prevents the observed increase in cell survival elicited by PGE₂. Additionally, actinomycin, an inhibitor of protein synthesis, did not alter the ability of PGE₂ to protect the cells from camptothecin induced apoptosis indicating that de novo protein synthesis is not required for the prostaglandin dependent protection.

Analysis of the AKT/protein kinase B phosphorylation status demonstrated that the treatment of the cells with PGE₂ results in a 4-fold increase in phosphorylation of serine 473 (Figure 6). Activation of AKT has been shown to decrease apoptosis through many different mechanisms. The specific mechanism by which PGE₂ stimulation of the EP4 receptor leads to survival is unclear, however, evaluation of the phosphorylation state of BAD after stimulation with PGE₂ indicates an increased level of phosphorylation at serine 136 ($\sim$ 3.5 fold by densitometry). The pharmacology of the phosphorylation of BAD is identical to that of the PGE₂-mediated survival response. PGE₂ and Prostaglandin E1-alcohol are effective while sulprostone and butaprost are not. Furthermore, PGE₂ dependent phosphorylation of pBAD136 is also blocked by pretreatment with EP4A (data not shown).

4. DISCUSSION

Using a well-characterized model of apoptosis, we have demonstrated that PGE₂ is capable of eliciting a survival response in Jurkat cells, and that the response in these cells is mediated by the EP4 receptor. Our data also demonstrates that PGE₂ signaling through the EP4 receptor results in activation of PI3Kinase /AKT signaling pathways that regulate cell survival. These data confirm a previous observation that EP4, in addition to EP2, can mediate a survival response and suggest that different downstream effector pathways are able to mediate cytoprotection based upon the particular EP receptor activated.

Camptothecin is a topoisomerase I inhibitor that is highly cytotoxic and is a potent anticancer chemotherapeutic agent. Jurkat cells have been shown to be particularly sensitive to camptothecin and cells that come in contact with this agent undergo a complete cessation of DNA synthesis followed by an increase in apoptosis(18). The data presented demonstrate that treatment of Jurkat cells with PGE₂ is able to protect cells from the apoptotic stimuli caused by camptothecin. This observation is consistent with a large body of evidence describing the pro-survival, anti-apoptotic effects of PGE₂. Our data demonstrate an 8-fold increase in caspase activity when compared to untreated cells and 40% apoptosis as judged by the population of cells with subdiploid DNA content by FACs analysis. Addition of PGE₂ prior to camptothecin treatment of the cells reduced apoptosis by 30-35%.

PGE₂ acts via four specific transmembrane G protein-coupled receptors designated EP1-4 (22). EP1 signals via increased intracellular Ca²⁺. EP3 inhibits the generation of cAMP, whereas EP2 and EP4 stimulate an increase in cAMP levels. Multiple EP3 isoforms have been described, which couple to different signaling pathways. Using EP-specific agonists and antagonists, we have addressed the involvement of EP receptors in PGE₂-induced signaling and protection from camptothecin-induced apoptosis expression in Jurkat cells. We found that neither the EP2 agonist, butaprost nor the EP3 agonist, sulprostone were able to substitute for PGE₂ in conferring protection from camptothecin-induced apoptosis. This is consistent with an earlier report suggesting that Jurkat cells lacked functional EP2 receptors, as butaprost was unable to elicit an elevation of cAMP. (16,17). Using Real-Time RT PCR we did not detect EP2 receptor mRNA as did a previous report that EP2 was undetectable in Jurkat cells by RT-PCR (16). The functional expression of EP3 receptors on Jurkat cells has been previously documented by a study that examined the role of PGE₂ in prolactin secretion(17). In that study, the EP1/3 agonist 17-phenol trinor PGE₂ was able to stimulate prolactin secretion. Our data confirm the presence of EP3 mRNA by Real-time PCR and western blotting in these cells; but demonstrate the inability of sulprostone to mimic the protective response to PGE₂, thus indicating that these receptors do not play a role in the cytoprotective effects of PGE₂. Our data however also confirm EP4 receptor expression, and that the specific antagonist EP4A blocked PGE₂-induced cytoprotection.

In many cell types, the protective PGE₂ response is mediated through the elevation of cAMP levels by activation of the EP2 receptor. Both G_is coupled EP2 and EP4 are capable of elevating cAMP in cells and elevation of intracellular cAMP levels by addition of exogenous cAMP analogs or treatment with forskolin is able to mimic the PGE₂ protective response in many cell types. The maximal level of PGE₂-stimulated cAMP formation in EP4 receptor-transfected cells was only ~20% that achieved in EP2 receptor-transfected cells, even though both receptors were expressed to nearly the same extent prior to agonist pretreatment (23). Recent studies suggest that not only are EP4 receptors less efficiently coupled to adenylate cyclase, but they have additional pathways of signal transduction that do not involve the activation of cAMP/PKA signaling (24). Moreover, there is an increasing body of evidence indicating that the G_s coupled EP4 receptor transduces signals through the PI3 kinase pathway to a greater degree than through activation of adenylate cyclase. In HEK cells transfected with EP4 receptor, PGE₂ activated Tcf/Lef signaling in a PI3 kinase -dependent manner(24). Additionally, PGE₂ stimulation of EP4 led to phosphorylation of the extracellular signal-regulated kinases (ERKs) via PI3 kinase dependent pathway and stimulation of EP4 receptors in LS-174 cells increased cell motility and proliferation in a PI3K-dependent manner(25). Our data suggest that the PGE₂ dependent survival response in Jurkat cells is mediated by activation of the EP4 receptor and downstream PI3/AKT kinase pathways. We have demonstrated that the PI3 kinase inhibitors, LY294002 and Wortmannin block the PGE₂ dependent increase survival in Jurkat cells whereas treatment with H89 a PKA specific inhibitor did not. Furthermore PGE₂ treatment of the cells results in increased phosphorylation of AKT kinase at serine 473. Inhibition of AKT activation (AKTi) also blocks the PGE₂ mediated increase in cell survival.

The exact mechanism by which EP4 is linked to PI3 kinase remains unclear. Murga et. al. have demonstrated the activation of both PI3K α and PI3K β through the $\beta\gamma$ heterodimer of the G-protein complex of the β_1 -adrenergic receptor (26). Activation of PI3 kinase has also been shown to be mediated through the alternate cAMP exchange protein, EPAC (27). Recently Fujino and Regan have reported that EP4 can activate PI-3 kinase through an inhibitory G-protein (*Mol Pharmacol* 69:5-10, 2006).

Activation of the AKT kinase is now recognized as a critical mechanism, associated with activation of multiple downstream pathways that increase cell survival. AKT dependent phosphorylation of p21, MDM2, BAD, AFX and FKHR all promote cell survival (28). In this report we have demonstrated that phosphorylation of BAD is stimulated by PGE₂ in Jurkat cells. BAD is an important proapoptotic member of the Bcl-2 family, which acts by inactivating antiapoptotic Bcl-2 family proteins (29,30). BAD exists in an inactive complex with the molecular chaperone 14-3-3 via the phosphorylation of four serine residues (Ser-112, -136, -155, and -170) (31). Following apoptotic stimuli, BAD is dephosphorylated, dissociated from 14-3-3, and translocated to the outer membrane of the mitochondria, where it subsequently dimerizes with Bcl-XL and promotes mitochondrial sequestration of BAD in the cytosol, preventing BAD from associating with and inactivating BCL-X_L. BCL-X_L inhibits the release of cytochrome C from the mitochondria and prevents the subsequent activation of caspase 9 resulting in inhibition of apoptosis.

There is an abundance of evidence supporting PGE₂ also functioning in tumor initiation and progression, as well as a mechanism of resistance to radiation treatment or chemotherapy. Increased survival of a transformed cell in response to PGE₂ stimulation is one potential mechanism by which prostaglandins may contribute to tumor initiation and progression. Our results demonstrate that EP4 in addition to EP2 can elicit a survival response and that the downstream mechanisms mediating the survival differ. Given the significance of increased COX-2 derived PGE₂ synthesis in many cancers, receptor specific targeted inhibition of PGE₂ mediated signaling pathways may provide an excellent strategy to enhance the efficacy of chemotherapeutic and chemopreventative agents.

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Figure 1.

a. Jurkat cells were treated with 25 μ M Camptothecin for 6 hours with or without 1 hour pretreatment with 2 nM PGE₂. Caspase-3/7 activity was assayed using a fluorescent substrate as described in Methods. Activity is expressed as Relative Fluorescence Units (RFLU). Data represent the mean $\pm$ standard error of 5 experiments in triplicate. B. Cells were treated as above and harvested after 12 hours, stained with propidium iodide for FACS analysis of apoptosis as described in Methods. Data are from representative experiment, N=3.

Figure 2.

EP receptor expression was assayed by (a) real time PCR or (b) Western blot as described in Methods. Western blots were quantitated by densitometry. Values for RNA and protein are expressed relative to EP4. Antibody specificity was confirmed (c) by pre-incubating diluted antibody with its corresponding blocking peptide. Data are from representative experiment, N=3.

Figure 3.

Cells were pretreated with EP agonist as indicated 1 hour before addition of 25uM camptothecin. The concentration of prostanoid added were: PGE₂, 5nM; Butaprost, 5uM; Sulprostone, 0.1uM; PGE1-alcohol, 5uM. Caspase activity was assayed 6 hours later as described in Methods. Caspase activity is expressed in relative fluorescence units. Data represent the mean ± standard error of 3 experiments in triplicate

Figure 4.

Cells were treated and caspase activity assayed as in Figure 1. When added, cells were treated with 0.1 μ M EP4A 1 hour before addition of 5nM PGE₂. Levels are expressed as percent caspase activity elicited by camptothecin. Data represent the mean $\pm$ standard error of 5 experiments in triplicate.

Camptothecin	+	+	+	+	+	+	+
PGE ₂	-	+	+	+	+	+	+
LY294002	-	-	+	-	-	-	-
Wortmannin	-	-	-	+	-	-	-
AKTi	-	-	-	-	+	-	-
H89	-	-	-	-	-	+	-
Actinomycin	-	-	-	-	-	-	+

Figure 5.

Cells were treated and caspase activity assayed as in Figure 1. When added, cells were treated with inhibitors 1 hour before addition of 5nM PGE₂. The concentration of inhibitors added were: LY294002, 10uM; Wortmannin, 0.1uM; AKTi, 10uM; H89, 0.1uM; actinomycin, 10ug/ml. Levels are expressed as percent caspase activity elicited by 25 uM camptothecin alone. Data represent the mean ± standard error of 3-5 experiments in triplicate.

A.

Fold increase
(normalized for load)

3.8 3.5

B.

pBAD

Fold increase
(normalized for load)

2.6 2.3 1.1 1.15

Figure 6.

Cells (2×10^6) were treated with 5nM PGE₂ or 5 uM PGE1-alcohol as in caspase experiments, washed with PBS and lysed with 50ul RIPA buffer. Proteins were isolated by PAGE using Invitrogen NU-PAGE system and blotted to nitrocellulose membrane. The blot was incubated and developed with phospho-specific antibody to either pBAD (ser-136) or pAKT (ser-473), stripped and reprobed with antibody to AKT protein. Data are from representative experiment, N=3.