Early de Novo Gene Expression Is Required for 15-Deoxy-Δ^{12,14}-prostaglandin J_2-induced Apoptosis in Breast Cancer Cells

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Cyclopentenone prostaglandin derivatives of arachidonic acid are potent inducers of apoptosis in a variety of cancer cell types. Several investigators have shown that the terminal derivative of prostaglandin J_2 (PGJ_2) metabolism, 15-deoxy-Δ^{12,14}-PGJ_2 (15dPGJ_2), induces apoptosis in breast cancer cells and is a potent activator of the nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPARγ), but 15dPGJ_2 effects can be mediated by PPARγ-dependent and PPARγ-independent mechanisms. Here we report that 15dPGJ_2 regulates early gene expression critical to apoptosis. Specifically, 15dPGJ_2 induces potent and irreversible S phase arrest that is correlated with expression of genes critical to cell cycle arrest and apoptosis, including the cyclin-dependent kinase inhibitor p21^WAF1/CIP1 (p21). Inhibition of RNA or protein synthesis abrogates apoptosis induced by 15dPGJ_2 in breast cancer cells but potentiates apoptosis induced by tumor necrosis factor-α or CD95/Fas ligand. Additionally, 15dPGJ_2 induces caspase activation that is blocked by peptide caspase inhibitors. These data show that de novo gene transcription is necessary for 15dPGJ_2-induced apoptosis in breast cancer cells. Critical candidate genes are likely to be revealed through analysis of differential cDNA array expression.

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EXPERIMENTAL PROCEDURES

Reagents and Cell Culture—15dPGJ_2 was purchased from Cayman Chemical (Ann Arbor, MI). 15dPGJ_2 is rapidly interconverted to a mixture of at least five active isomers (29). Actinomycin D, cycloheximide, 4′, 6-diamidino-2-phenylindole dihydrochloride, TNFα, staurosporin, and CD95/Fas ligand were purchased from Immunotech (St. Louis, MO). Caspase inhibitors were purchased from Calbiochem. MDA-MB-231 breast cancer epithelial cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1% penicillin, 1% streptomycin, and 1% L-glutamine (Life Technologies, Inc.). For cell synchronization experiments, cells were synchronized as described previously (30).

Flow Cytometry for Markers of Apoptosis—Cells were incubated with or without actinomycin D (1 μg/ml) or cycloheximide (2 μg/ml) for 1 h and then exposed to 15dPGJ_2 (10 μM) or vehicle (ethanol). After 36 h, cells were collected by trypsinization and pelleted, and the percentage of cells undergoing apoptosis was determined by flow cytometry using a TACs annex V-fluorescein isothiocyanate kit (Trevegin, Gaithersburg, MD) according to the protocol of the manufacturer.

Histochemistry and Immunofluorescence—Cells were incubated with or without actinomycin D (1 μg/ml) or cycloheximide (2 μg/ml) for 1 h and then exposed to 15dPGJ_2 (10 μM) or vehicle (ethanol). After 36 h, cells were stained with annexin V-fluorescein isothiocyanate monochondrular antibody (Roche Molecular Biochemicals) followed by overnight incubation with 0.1 μg/ml 4′, 6-diamidino-2-phenylindole dihydrochloride in 10% formaldehyde. Dual stained cells were washed and mounted under a glass coverslip, and digital images were obtained using a Zeiss Axiplan-2 epifluorescence microscope equipped with UV excitation filters and a Spot digital camera.

Differential Display Analysis—Cells were incubated with or without 15dPGJ_2 (10 μM) for 2 h. Total RNA was isolated using Trizol Reagent (Life Technologies, Inc.), and 1.0 μg of total RNA was used to create radiolabeled cDNA by RT-PCR and hybridized to a Human Broad Coverage cDNA Array 1.2 (CLONTECH, Palo Alto, CA) according to the protocol of the manufacturer. Relative gene expression level and the mean from three separate experiments were determined using AtlasIm-

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The abbreviations used are: PGJ_2, prostaglandin J_2; 15dPGJ_2, 15-deoxy-Δ^{12,14}-prostaglandin J_2; PPAR, peroxisome proliferator-activated receptor; p21, p21^WAF1/CIP1; p27, p27^KIP1; AA, arachidonic acid; COX-2, cyclooxygenase-2; (15S)-HETE, (15S)-hydroxyeicosatetraenoic acid; NFκB, nuclear factor κB; TNFα, tumor necrosis factor-α; RT, reverse transcription; PPB, PPARγ response element; ZVAD-fmk, N-benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone.
Western Blot Analysis—MDA-MB-231 cells were incubated with or without actinomycin D (1 µg/ml) or cycloheximide (2 µg/ml) for 1 h and then exposed to 15dPGJ2 (10 µM) or vehicle (ethanol). After 2 h, total protein was isolated in lysis buffer, and 50 µg of total protein was separated by gel electrophoresis in 10–20% SDS-polyacrylamide precast gels (Bio-Rad) and transferred to polyvinylidene difluoride membranes (Bio-Rad). Antibodies to p21Waf1/Cip1, p27Kip1, and procaspase-3 were from Santa Cruz Biotechnologies (Santa Cruz, CA). Bands were visualized using ECL-Plus (Amersham Pharmacia Biotech) and Kodak BioMax film (Eastman Kodak Co.).

Northern Blot Analysis—MDA-MB-231 cells were incubated with or without actinomycin D (1 µg/ml) or cycloheximide (2 µg/ml) for 1 h and then exposed to 15dPGJ2 (10 µM) or vehicle (ethanol). After 2 h, total RNA was isolated in Trizol reagent (Life Technologies, Inc.), and 10 µg was separated by gel electrophoresis and transferred to ZetaProbe GT membranes (Bio-Rad). cDNA probes to human p21 and β-actin were amplified by RT-PCR using Gene Amp Gold RNA PCR kit (PerkinElmer Life Sciences), cloned into TOPO cloning vectors (Invitrogen, Carlsbad, CA), and labeled and hybridized using a Strip-EZ RNA kit (Ambion, Austin, TX). Autoradiograph images were obtained with BioMax film (Kodak) by overnight exposure at −70 °C.

Caspase Activity Assays—Cells were incubated with 15dPGJ2 (10 µM) with or without the indicated caspase inhibitors (10 µM). After 36 h, phosphatidylserine translocation was determined by flow cytometry as described above, and immunoblots for procaspase-3 (Santa Cruz Biotechnologies) expression was performed as described above.

RESULTS

Inhibition of RNA or Protein Synthesis Blocks 15dPGJ2-induced Apoptosis—Previous experiments in our laboratory showed that MDA-MB-231 cells undergo apoptosis as early as 8 h after exposure to exogenous 15dPGJ2 (2). Here we investigated the minimum amount of exposure time necessary to induce apoptosis. Cell growth was markedly inhibited after cells were incubated with 15dPGJ2 for as little as 2 h (Fig. 1A). Incubation of cells with 15dPGJ2 or the phosphatase inhibitor staurosporin for 36 h induced marked apoptosis that was blocked by actinomycin D and cycloheximide, respectively (Fig. 1B). However, TNFα and CD95/Fas ligand induced apoptosis only when RNA and protein synthesis was blocked. NFκB mediates a mechanism of protection via expression of cytoprotective genes, but TNFα pretreatment of breast cancer cells does not rescue cells from 15dPGJ2-induced apoptosis (data not shown).

Further investigation showed that treatment of cells with 15dPGJ2 for as little as 5 h irreversibly induced apoptosis, which was markedly inhibited by pretreatment with actinomycin D or cycloheximide (Fig. 1C). Additionally, phosphatidylserine translocation and nuclear condensation, hallmarks of apoptosis, were attenuated when RNA or protein synthesis was blocked (Fig. 1D) in 15dPGJ2-treated cells. Together these data show that early de novo transcription of genes is required for 15dPGJ2-induced apoptosis.

Genes Regulated by 15dPGJ2—Recently DuBois and colleagues (33) investigated the expression of PPARγ target genes in colon cancer at 24 h and 6 days. Our data suggested that critical genes are transcribed much earlier. We investigated the 15dPGJ2-mediated expression of genes critical to apoptosis by differential display analysis at 2 h (Table I). The expression of gene products encoding proteins involved in cell cycle arrest and apoptosis, including Bag-1, a promoter enhancer and Bcl-2 binding protein; the cytosolic protease caspases 3, 4, and 8; the transducer and activator of transcription 1/EGR1, AP-1, and e-Jun; antioxidative genes like hEGR1, AP-1, and c-Jun; and genes involved in DNA maintenance and repair, including ERCC1, Rad52, Rad23A, and apoptosis, including Bag-1, a promoter enhancer and Bcl2-
induced gene transcription, caspase activation is induced that cannot be completely blocked by an individual caspase inhibitor.

**DISCUSSION**

The cyclopentenone prostaglandins possess potent antiproliferative and antitumor activities, but their mechanisms of action are complex and not well understood. Recently it was shown that 15dPGJ2 induces intracellular oxidative stress in human neuroblastoma cells and hepatic myofibroblasts (38, 39) and that 15dPGJ2 induces expression of antineoplastic enzymes, such as glutathione S-transferases (40). Additionally, Fitzpatrick and colleagues (37) showed that 15dPGJ2, the immediate precursor to 15dPGJ2, inhibits isopeptidase activity of the proteosome pathway. We have shown that 15dPGJ2 induces important and seemingly opposite biological responses, including proliferation, differentiation, and apoptosis in breast cancer cells and that these phenotypes correlate with increasing levels of PPRE-mediated transcription (1, 41). The studies presented here show that 15dPGJ2-induced apoptosis in breast cancer cells requires the rapid synthesis of new gene products (>5 h after exposure) that irreversibly leads to apoptosis. Differential DNA array analysis suggests candidate genes including p21 and p27, which are potentially critical to this process given that 15dPGJ2 induces an S phase arrest.

In addition to cell cycle arrest, p21 and p27 may play a critical role in apoptosis. Growth factor withdrawal leads to a proapoptotic feedback loop involving p21, p27, and caspase-3 in human endothelial cells (42, 43). Furthermore, caspase-3-mediated cleavage of p21 is an early event after DNA damage (44). Moreover, dominant negative mutants of p21 abrogated apoptosis in these studies. Together these reports suggest that transcriptional activation of p21 and p27 followed by caspase-3-mediated cleavage represents a potential mechanism of action for 15dPGJ2. However, our data suggest the relationship is not so straightforward, at least for p21. Actinomycin clearly blocks 15dPGJ2-induced apoptosis but does not reduce early 15dPGJ2-induced p21 protein levels. One explanation for this may be that arachidonic acid and many of its metabolites

### Table I

**Summary of gene expression after exposure of MDA-MB-231 cells to 15dPGJ2 for 2 h**

| GenBank Accession number | Gene Name | Fold Increase |
|--------------------------|-----------|---------------|
| X02541 early growth response protein 1 (EGR1); transcription factor | 37.96 |
| M62629 factor ETR103; KROKOG4 zinc finger protein 225; AT226 | 7.20 |
| X33405 heme oxygenase 1 (HO1); HMOX1 | 2.00 |
| P01957 heat shock 70-kDa protein (heat shock 70-kDa protein B) | 4.60 |
| S65717 BCL-2 binding shahogangine-1 (BAG-1); glucose oxidase | 4.60 |
| J04088 DNA topoisomerase II alpha (TOP2A) | 4.11 |
| X81376 5-ICE-LAP5; MORT1-associated CED-3 homolog (MACH); | 3.98 |
| U65203 FACD-homologous (CED3-like protein) (FACD-like) | 3.98 |
| US9143 ICE; FLICE; apoptotic cytochrome c (MTH1); | 3.98 |
| S40706 GADD153; DNA damage-inducible transcript 3 (DIT3); | 3.98 |
| S62158 GSHB homologous protein (CHOP); | 3.98 |
| M96994 purine-rich single-stranded DNA-binding protein alpha | 3.17 |
| M11717 70-kDa heat shock protein 1 (HSPT1); HSPA1 | 2.49 |
| L92292 catalase kinetic 1 (CLO1) | 2.35 |
| M333374 NAD(P)H-ubiquinone oxidoreductase B1 subunit; complex I | 2.34 |
| L12290 heregulin-beta3; glial growth factor; neurotrophin | 2.29 |
| D60781 bone morphogenetic protein 4 (BMP4); bone | 2.29 |
| M24980 CYP-450 aromatic hydrocarbon receptor (CYP2B) | 2.29 |
| AP012108 heat shock protein 40 (HSPA4) | 2.18 |

| Gene Name | Fold Decrease |
|-----------|---------------|
| monocyte chemotactic protein 1 precursor (MCP1); | - |
| M37719 monocyte chemotactic and activating factor (MCAF); | - |
| A0033576 monocyte secretory protein 1; monocyte chemotactic protein 1 precursor (MCP1); | - |
| M24545 protein 1 (H11); small inducible cytokine A2 (SCYA2); | 1.50 |
| D15431 hepatoma-derived growth factor (HDGF); | 1.50 |
| leukocyte adhesion glycoprotein; LFA-1 alpha subunit | 1.50 |
| praeceptor; leukocyte-function-associated molecule 1 alpha | 1.50 |
| A003356 ch5h; CD11A alpha; integrin alpha L (ITGAL); | 1.50 |
| U04166 E1B protein | 1.50 |
| tyrosine-protein kinase receptor UFO precursor; act | 1.51 |
| M76125 oncogene | 1.51 |
| D21235 HHR23A; UV excision repair protein RAD23A | 1.53 |
| intercellular adhesion molecule 1 precursor (ICAM1); | 1.53 |
| J03312 group minio receptor; CD54 antigen | 1.57 |
| cyclin-dependent kinase inhibitor 6 (CDKN6); | 1.67 |
| cyclin-dependent kinase-associated dual specificity phosphatase; | 1.67 |
| kinase-associated phosphatase (KAP); cyclin-dependent kinase | 1.67 |
| L52876 interactor 1 (CD1) | 1.60 |
| X54941 cyclin-dependent kinase regulatory subunit 1 (CKS1) | 1.61 |
| M13194 DNA excision repair protein ERCC1 | 1.65 |
| M61176 brain-derived neurotrophic factor (BDNF) | 1.67 |
| U06540 p53Cdk | 1.76 |
| Y75342 sphin proton-conogen | 1.91 |
| U12134 DNA damage repair & recombination protein 52 (RAD52); | 2.05 |
| M23997 coloradial mutant protein (CCM); | 2.10 |
| AF040105 FACL growth-related 5-myo-regulatory gene | 2.18 |
| M73077 globulinoid receptor trypsin factor 1 | 2.28 |
| U43741 breast cancer type 2 susceptibility protein (BRCA2); | 2.50 |
| M34286 cAMP response element-binding protein (CREB1) | 2.60 |
| AF019686 DAXX | 2.69 |
| U13821 ionizing radiation-resistance-conferring protein | 2.69 |
| X834544 monocyte-activated protein kinase p38 (MAP kinase p38); | 3.10 |
| c75159 cytokine suppressor anti-inflammatory drug binding protein | 3.10 |
| L35253 C-75159 binding protein, CSP; MAP-interacting protein | 3.27 |
| L35285 protein C75159 | 3.27 |
| DNA (gase; I); polyoxylhexosamine synthase (ATP) | 3.34 |
| M00670 growth arrest; DNA-damage-inducible protein 45 beta | 7.11 |

| Gene Name | Fold Decrease |
|-----------|---------------|
| (GADD45 beta) | - |
increase protein kinase C activity (45), which has been shown to stabilize p21 mRNA (46). Further research is needed to reconcile these contradictory data.

Recent investigations have shown that PPARγ agonists differentially regulate genes associated with cell growth and differentiation. 15dPGJ2 negatively regulates myogenesis in part by inhibition of MyoD gene expression (47), and DuBois and colleagues (33) used microarray technology to show that inhibition of MyoD gene expression (47), and DuBois and colleagues (33) used microarray technology to show that inhibition of MyoD expression is increased by 15-deoxy-Δ12,14-prostaglandin J2 and blunted by actinomycin D or cycloheximide. A, MDA-MB-231 cells were synchronized and treated with or without 15dPGJ2, and cell cycle progression was determined by flow cytometry. B, total protein was isolated from synchronized cells at the indicated times, and the expression of p21 and p27 was determined by immunoblotting. C, total protein from asynchronous cells treated with the indicated compounds was isolated at 9 h, and the expression of p21 and p27 was determined by immunoblotting. D, total RNA from asynchronous cells treated with the indicated compounds was isolated at 2 h, and the expression of p21 was determined by Northern blot analysis. The expression of β-actin was used as a control. The lack of new p21 mRNA expression during treatment with 15dPGJ2 and actinomycin suggests that p21 protein detected under similar conditions (C) is from preformed p21 mRNA (see “Discussion”). Chx, cycloheximide; Act, actinomycin; 15d, 15dPGJ2.

Regulation of arachidonic acid (AA) metabolism is critical to the growth and survival of all cell types. 15dPGJ2 inhibits transcriptional activation of COX-2 by a negative feedback loop mediated through PPARγ (28, 48), which may lead to increased intracellular levels of free AA. Blocking arachidonate-phospholipid remodeling, by inhibition of CoA-independent transacylase (30, 49, 50) or inhibition of fatty acid-CoA ligase 4 (51), may produce nonenzymatic oxidized AA metabolites via increased levels of unesterified AA, which results in apoptosis of several cancer cell types. In addition, hexadecyl azelaoyl phosphatidylcholine, a novel phospholipidase A2 digestion product of alkyl phosphatidylcholines, a novel phospholipid, has been reported to induce apoptosis of prostate adenocarcinoma (56), and addition of exogenous (15S)-HETE induces apoptosis of prostate cancer cells (57). Inhibition of COX-2 by cyclopentenone prostaglandins with coordinate increases in intracellular oxidative stress could lead to the generation of oxidized AA metabolites as endogenous activators of PPARs or other transcription factors, creating a positive feedback loop of PPAR activation resulting in expression of gene products critical to apoptosis. This may account for the fact that 15dPGJ2 is a far more potent activator of PPRE-mediated transcription than any other PPARγ agonist.

While many of the hypotheses presented here are being tested in our laboratory, deeper understanding of how AA metabolites induce cancer cell growth or death is essential. Common chemotherapeutic drugs including alkylating agents and nucleoside analogues work by blocking cellular replication at the level of DNA or interfere with ubiquitous cellular process.

FIG. 2. p21<sub>casp-1<sub>15dPGJ2</sub></sub> and p27<sub>casp-1<sub>15dPGJ2</sub></sub> expression is increased by 15-deoxy-Δ12,14-prostaglandin J2 and blunted by actinomycin D or cycloheximide. A, MDA-MB-231 cells were synchronized and treated with or without 15dPGJ2, and cell cycle progression was determined by flow cytometry. B, total protein was isolated from synchronized cells at the indicated times, and the expression of p21 and p27 was determined by immunoblotting. C, total protein from asynchronous cells treated with the indicated compounds was isolated at 9 h, and the expression of p21 and p27 was determined by immunoblotting. D, total RNA from asynchronous cells treated with the indicated compounds was isolated at 2 h, and the expression of p21 was determined by Northern blot analysis. The expression of β-actin was used as a control. The lack of new p21 mRNA expression during treatment with 15dPGJ2 and actinomycin suggests that p21 protein detected under similar conditions (C) is from preformed p21 mRNA (see “Discussion”). Chx, cycloheximide; Act, actinomycin; 15d, 15dPGJ2.

FIG. 3. 15-Deoxy-Δ12,14-prostaglandin J2-induced apoptosis requires caspase-3. A, MDA-MB-231 cells were exposed to 15dPGJ2. Total protein was isolated at the indicated times and analyzed for expression of procaspase-3 (Pro-Cas-3) by immunoblotting. B, MDA-MB-231 cells were grown in the presence of 15dPGJ2 (15d) with or without ZVAD-fmk. Total protein was isolated after 36 h and analyzed for expression of procaspase-3 by immunoblotting. C, MDA-MB-231 cells were grown in the presence of 15dPGJ2, with or without the pan-caspase inhibitor ZVAD-fmk, the caspase-8 inhibitor IETD-fmk, or the caspase-9 inhibitor LEHD-fmk. After 36 h, the percentage of cells undergoing apoptosis was determined by flow cytometry.
such as microtubule lysis. This strategy reduces cell proliferation, but relapse and the development of resistance to chemotherapy suggest that cancer cells are not eradicated. The data presented here demonstrate that gene expression may be required for the induction of apoptosis and thus eradication of some cancer cell types. In addition, potent bioactive derivatives of AA, such as cyclopentenone prostaglandins, acting through multiple pathways may represent a promising class of therapeutic molecules for the treatment of cancer. However, the potential of these compounds to act as proliferators of tumorigenesis via PPARγ (58, 59) suggests that it is critical to further clarify the mechanisms that regulate the antiproliferative and proapoptotic activities of these compounds. The data presented here strongly suggest that new gene synthesis is required for 15dPGJ2-induced apoptosis. Whether this is mediated via PPARγ or some other critical regulator of gene synthesis remains to be defined.

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