Resolvin D2 Supports MCF-7 Cell Proliferation via Activation of Estrogen Receptor

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ABSTRACT

Inflammation has been implicated in tumor initiation, angiogenesis, and metastasis, and linked to the development of more aggressive, therapy-resistant estrogen receptor (ER)–positive breast cancer. Resolvin D2 (RvD2) is a potent anti-inflammatory lipid mediator. As RvD2 may be synthesized within breast tumors by both tumor cells and the surrounding stroma cells and is present in plasma at bioactive concentrations, we sought to characterize the impact of RvD2 on cell processes underlying breast tumor growth and spread. Trypan-blue exclusion, transfection with estrogen response element (ERE) reporter, real-time quantitative polymerase chain reaction, competitive radioligand binding assays, Western blotting, and immunofluorescence were the techniques used. Unexpectedly, whereas RvD2 (10–1000 nM) supported the proliferation of the ER-positive breast tumor (MCF-7) cells, it did not affect the ER-negative MDA-MB-231 cell number. The proliferative effect of RvD2 in MCF-7 cells was attenuated by the ER antagonist ICI 182,780 (7H-[9-[4-(4,5,5,5-pentafluoropentyl)sulfanyl][4-nonyl]jesta-1,3,5(10)-trien-3,17β-diol). Furthermore, RvD2 increased ERE transcriptional activity in a number of ER-positive breast and ovarian tumor cell lines. This activation was also inhibited by ICI 182,780. RvD2 altered the expression of a subset of estrogen-responsive genes. Although binding experiments showed that RvD2 did not directly compete with H-[9]-estradiol for ER binding, prior exposure of MCF-7 cells to RvD2 resulted in a significant reduction in the apparent cytosolic ER density. Confocal immunocytochemistry and Western blotting studies showed that RvD2 promoted nuclear localization of EREs. These observations indicate that RvD2 displays significant but indirect estrogenic properties and has the potential to play a role in estrogen-dependent breast cancer progression.

INTRODUCTION

Despite the substantial advances in treatment options and the overall better survival rate in recent years, breast cancer remains the most common cancer in women (Jacobs and Finlayson, 2011). In addition to genomic instability and other factors specific to cancer cells, inflammation is now considered a hallmark of cancer and can play a role in virtually all aspects of tumor biology, including initiation, promotion, angiogenesis, and metastasis (Hanahan and Weinberg, 2011). Chronic inflammation that precedes tumor development, tumor-associated inflammation, and therapy-induced inflammation are the main types of inflammation involved in tumorigenesis and cancer development (Jiang and Shapiro, 2014). Some risk factors for breast cancer, such as menopause, increased age, and obesity, are associated with systemic inflammation, as indicated by high levels of circulating inflammatory cytokines (Bruunsgaard et al., 2001; Pfeilschifter et al., 2002; Ouchi et al., 2011). An inflammatory tumor microenvironment consists of infiltrating immune cells and activated fibroblasts, both of which can secrete cytokines, chemokines, and growth factors, as well as DNA-damaging free radicals and oxidants. Tumors themselves are able to both generate and respond to inflammatory microenvironments (Baumgarten and Frasor, 2012).

Resolution of inflammation, previously believed to take place passively, is now considered an actively programmed process distinct from anti-inflammatory processes, enabling the host tissue to return to homeostasis (Serhan et al., 2007). Specialized proresolving lipid mediators (SPMs), including lipoxins, resolvins, and maresins are new families of endogenously synthesized chemical autacoids identified by Serhan and collaborators, which have been characterized for
their anti-inflammatory and proresolving properties (Serhan, 2007). In inflamed sites, neutrophils can interact with neighboring cells, such as other leukocytes, platelets, endothelial cells, mucosal epithelial cells, and fibroblasts, in their immediate vicinity and acquire the ability to produce SPM (Serhan et al., 2008). Resolvin D2 (RvD2) is a member of this family, biosynthesized from the polyunsaturated fatty acid docosahexaenoic acid by the sequential actions of 15- and 5-lipoxygenases (Serhan and Petas, 2011). Biologically active concentrations of RvD2 can be readily measured in human blood after n-3 fatty acid supplementation (Mas et al., 2012).

We have previously demonstrated that a member of this family, lipoxin A4, stimulates the proliferation of estrogen receptor (ERα)-positive MCF-7 and ERα-negative MDA-MB-231 breast cancer cell lines via formyl peptide receptor 2 signaling (Khau et al., 2011). Therefore, we hypothesized that the persistence of inflammation is accompanied by persistent production of SPM, which may stimulate breast cancer growth. Given that both lipoxin A4 and RvD2 share a significant structural homology (trihydroxytetraenes), which is a feature of all eicosanoids of this class (Serhan et al., 2008), and that the enzymes involved in RvD2 synthesis are expressed in normal and tumor mammary tissues (Jiang et al., 2006a,b), we sought to gain further insights into the impact of the SPM RvD2 on breast tumor cell functions related to growth and dissemination.

Materials and Methods

Cell Culture and Treatment Preparations. The MDA-MB-231 cell line was grown in phenol red–free RPMI 1640 (Invitrogen, Carlsbad, CA) containing 10% (v/v) heat-inactivated fetal calf serum (Sigma-Aldrich, Castle Hill, NSW, Australia), 2 mM l-glutamine (Sigma-Aldrich, St. Louis, MO), 1 mM nonessential amino acids (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), 100 U/ml penicillin G, 100 µg/ml streptomycin (CSL Biosciences, Parkville, VIC, Australia), 15 mM HEPES, and 0.2% (v/v) sodium bicarbonate. MCF-7, T47D, BT474, and SKOV3 cells were cultured in phenol red–free Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented identically to RPMI, already described herein. Cells were passaged every 3 to 4 days at a density of 3 × 10⁵ cells per 75-cm² flask and maintained at 37°C in 5% CO₂. For the experiments, the cells were seeded at 50,000 cells/well in 24-well plates and incubated in serum-free DMEM medium containing, in addition to 0.25% (v/v) bovine serum albumin (BSA), all the mentioned supplements for 24 hours before incubation with different treatments of interest as specified to follow.

All compounds were dissolved in 100% dimethylsulfoxide to a stock concentration of 10 mM, stored at −20°C, and diluted in fresh medium just before use. To ensure stability, RvD2 stock was stored at −80°C, and each aliquot was thawed and discarded immediately after use. Vehicle solutions were used for the respective control treatment. The conditions for each treatment, including 17β-estradiol (E₂) and RvD2, was chosen based on preliminary studies and previous publications (Hughes et al., 2002; Miyahara et al., 2013).

Cell Enumeration. Viable MCF-7 and MDA-MB-231 cells were identified by trypan blue (BDH/Merck, Kilsyth, VIC, Australia) exclusion and enumerated (by an operator blinded to treatment) with the aid of a hemocytometer 24–72 hours after treatment with 5% fetal calf serum (v/v) or different concentrations of RvD2, obtained by total chemical synthesis (Li et al., 2013). In separate experiments, MCF-7 cells were treated with the ER antagonist ICI 182,780 (7α-[4,4,5,5-pentafluoropentyl]bulsulfonyl[oxetyl]estr-1,3,5(10)-triene-3,17β-diol; 100 nM; Sigma-Aldrich) for 30 minutes before E₂ (10 nM) or RvD2 (100 nM) incubation to evaluate the potential involvement of ER. To investigate whether the proliferative effect of RvD2 involves activation of the signaling pathways that are known to mediate cell proliferation or survival, MCF-7 cells were incubated with inhibitors of extracellular signal–regulated kinase 1/2 (ERK1/2) (PD98059 [2-(2-amino-3-methoxyphenyl)-4-I-1-benzopyran-4-one] and U0126 [1,4-diamino-2,3-dicyano-1,4-bis[2-amino phenylthio]butadiene]) (Torrisi, Bristol, UK), phosphatidylinositide 3-kinase (PI3K) (LY294002 [2-(4-morpholinyl)-8-phenyl-1-(4H) benzopyran-4-one hydrochloride]) (Calbiochem/Merck, Kilsyth, VIC, Australia), or p38MAPK (SB202190 [4-(4-fluorophenyl)-5(4-pyridyl)-1H-imidazol-2-yl]phenol) and SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5(4-pyridyl)-1Himidazol] (Calbiochem/Merck) was added 30 minutes before RvD2 treatment.

Estrogen Response Element–Secreted Human Placental Alkaline Phosphatase Luciferase Reporter Construct Transfection. MCF-7, T47D, BT474, and SKOV3 cells were seeded in a penicillin/streptomycin-free medium for 24 hours. The estrogen response element (ERE)–secreted human placental alkaline phosphatase (SEAP) reporter construct (BD Biosciences/Clontech, Palo Alto, CA) (300 ng/well) was incubated with pGL3 Luciferase control vector (Promega, Madison, WI) (50 ng/well, to account for transfection efficiencies) and 1.0 µl of LipofectAMINE 2000 reagent (Invitrogen) in a volume of 100 µl/well of OptiMEM for 30 minutes at room temperature. Cells were then transfected for 6 hours, washed once with sterile phosphate-buffered saline (PBS) and incubated with 1 ml of serum-free DMEM. The next day, the cells were treated with RvD2 (10–1000 nM) or estradiol (1 nM) for 48 hours. Supernatants were collected at the end of each experiment and assessed for SEAP content using a chemiluminescence kit (Roche Applied Science, Castle Hill, NSW, Australia). At the conclusion of each experiment, cells were lysed in 25 mM Tris-phosphate, pH 7.8, lysis buffer containing 10% glycerol, 1% Triton X-100, 1 mg/ml BSA, 2 mM EDTA, 2 mM dithiothreitol (DTT). To assess luciferase activity, 50 µl of each cell lysate was transferred to a black and white 96-well plate and 25 µl of luciferin substrate (Promega) and reagent buffer (20 mM Tris-base PH 7.8, 33.3 mM DTT, 8 mM MgCl₂, and 0.13 mM EDTA) was added. Chemiluminescence for both secreted alkaline phosphatase and luciferase assays was measured by Topcount (PerkinElmer, Glen Waverley, VIC, Australia).

Reverse-Transcription Quantitative Polymerase Chain Reaction. To quantify gene expression, cells were treated for 4 to 24 hours with 0.1 nM E₂ or 0.1–1 µM RvD2 in the presence and absence of 100 nM ICI 182,780 or 10 µM LY 294002. Total RNA was extracted using trizol reagent according to the manufacturer’s instructions, reverse-transcribed into cDNA, using random primers for subsequent analysis by reverse transcription-polymerase chain reaction using the following thermal protocol: 97°C (60 minutes), 95°C (5 minutes), and 4°C (5 minutes). Transcript levels of genes of interest were assayed by reverse transcription-polymerase chain reaction with predeveloped assays (Applied Biosystems, Mulgrave, VIC, Australia) and Platinum SYBR Green qPCR Supermix-UDG (Invitrogen, Mulgrave, VIC, Australia) using the ABI Prism 7900 HT sequence detection system (Applied Biosystems). Cycle numbers to reach threshold (Cₚ) values for each reaction were determined using SDS 2.0 software (Applied Biosystems). All Cₚ values were normalized to the 18S rRNA Cₚ value as an internal control. Primer sequences used in this study are included in Table 1. They were obtained either from published references as annotated or designed using Primer Express software (Applied Biosystems) with mRNA sequences from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Radioligand Binding Assays Using [³H]E₂. Intact cells were treated with unlabeled E₂ or RvD2 for 30 minutes before incubating them with 0.1 nM [2,4,6,7-³H]E₂ (89.2 Ci/mmol; PerkinElmer) for 1 hour at 37°C in humidified air with 5% CO₂. To determine nonspecific binding, 100-fold excess of unlabeled E₂ was added. Ethanol was used as a negative control. Cells were then washed three times with ice-cold PBS to eliminate free and bound label; cellular [³H]E₂ was then extracted with 500 µl of 80% EtOH for 30 minutes at room temperature. The supernatant was mixed with 4 ml of Optiphase Hisafe 2 scintillation fluid (PerkinElmer) and the tritium counted. Cytosol from...
MCF-7 cells was prepared, and binding assays were performed as previously described (Hughes et al., 2002). In brief, MCF-7 cytosol was incubated with 0.2 nmol/l [3H]E2 in a 10 mmol/l Tris buffer (pH 7.4; 1.5 mmol/l EDTA, 10% w/v glycerol) in the presence and absence of 1 μM ICI 182,780 to define nonspecific binding. Increasing concentrations of either E2 or RvD2 were added to cytosol containing 0.2 nmol/l [3H]E2 to determine IC50 values for displacement of [3H]E2.

**Immunofluorescence.** Cells were seeded at 25,000 cells per chamber in an eight-chamber cell culture slide (Nunc, Roskilde, The Netherlands), allowed to attach for 24 hours, starved in DMEM containing 0.25% BSA for a further 24 hours, and then treated with 10 nM E2 or 1 μM RvD2 for 30 minutes. Cells were fixed in 10% v/v neutral-buffered formalin for 10 minutes, washed in PBS, and incubated with the primary antibody against ERα (ER rabbit polyclonal #sc-20; Santa Cruz Biotechnology, Dallas, TX) overnight at 4°C. Slides were washed twice with PBS and incubated with fluorescein isothiocyanate–conjugated secondary antibody for 1 hour at room temperature before nuclear staining using DAPI (4’,6-diamidino-2-phenylindole) (Santa Cruz Biotechnology) and coverslipping using fluorescence antifade mounting medium (DAKO, Braeside, VIC, Australia). Fluorescence microscopy (Zeiss Meta, North Ryde, NSW, Australia) was used to confirm colocalization of DAPI and ERα immunoreactivity.

**Subcellular Fractionation.** Cells were lysed in lysis buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mmol/l EDTA, and 1 mM EGTA) containing protease inhibitor cocktail and 1 mM DTT. To obtain the nuclear pellet, the lysates were passed 10 times through a 25-gauge needle and then centrifuged at 720g for 5 minutes. The nuclear pellet was then washed with lysis buffer, passed 10 times through a 25-gauge needle, and centrifuged again at 720g for 10 minutes. The nuclear pellet was then suspended in lysis buffer containing 10% (v/v) glycerol and 0.1% (w/v) sodium dodecyl sulfate and sonicated for 3 seconds. The supernatants (cytosolic fraction) from the first centrifugation were recentrifuged at 10,000g for 10 minutes, and the resulting supernatants were transferred into fresh tubes and centrifuged again at 100,000g for 1 hour to pellet cell membranes.

**Western Blot Analysis.** MCF-7 cells were seeded in six-well plates at 200,000 cells/well for 24 hours and incubated with serum-free media. After 24 hours of serum deprivation, cells were treated with 10 nM E2 or 1 μM RvD2 for 30 minutes. Cells were then washed twice in ice-cold PBS and lysed on ice for 20 minutes in lysis buffer containing NaCl (100 mM), Tris-Cl (10 mM) pH 7.5, EDTA (2 mM),

<table>
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<tr>
<th>Table 1</th>
<th>Primer sequences for reverse-transcription polymerase chain reaction</th>
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<tr>
<td>Gene</td>
<td>Forward Primer</td>
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<tr>
<td>18s</td>
<td>CGCCGCTAGAGGTGAAT</td>
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<tr>
<td>GREB1</td>
<td>GAGGATGTGGGAGGACCC</td>
</tr>
<tr>
<td>PGR</td>
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<tr>
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<tr>
<td>TGFβ2</td>
<td>GGGCACCTGGGAAACTGCAC</td>
</tr>
<tr>
<td>TGFβ3</td>
<td>CTGTCCTGTAGGTGGCTTGTG</td>
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**TGF,** transforming growth factor.

![Fig. 1. Effect of RvD2 on MDA-MB-231 and MCF-7 cell numbers. After incubation with serum-free media for 24 hours, MDA-MB-231 cells (A) and MCF-7 cells (B and C) were exposed to increasing concentrations of RvD2 (10–1000 nM) or fetal calf serum (FCS) (5%/v/v) for 24–72 hours. In separate experiments, MCF-7 cells were incubated with RvD2 (100 nM) or E2 (10 nM) for 48 hours in the presence or absence of the estrogen receptor antagonist ICI 182,780 (100 nM) (D). Numbers of viable cells were then counted. Data represent means ± S.E.M. (n = 6–10 for A, B, and D, and n = 1 for C, each determined in triplicates). **P < 0.001; ***P < 0.0001 versus vehicle control (B); ##P < 0.05, ####P < 0.0001 versus E2 and RvD2 treatments, respectively.**
deoxycholate (0.5% w/v) with phosphatase inhibitor cocktail (1% v/v) (Sigma-Aldrich), protease inhibitor (1% v/v) (Sigma-Aldrich), and MgCl₂ (1 mM) added immediately to the buffer before use. Cells were removed from the plastic dish by scraping, transferred to microfuge tubes, and centrifuged at 10,000g for 10 minutes at 10°C, after which supernatants were collected and protein content was determined by Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). Proteins were resolved electrophoretically on 12% SDS-PAGE and transferred onto nitrocellulose membranes (Amersham/GE Health Care, Rydalmere, NSW, Australia) for Western blotting. Membranes were blocked in 5% (w/v) skim milk for 1 hour at room temperature and then incubated overnight with anti-ERα rabbit polyclonal antibody (#sc-20; Santa Cruz Biotechnology) at 4°C. β-Actin (mouse monoclonal: Abcam, Cambridge, UK) and Lamin A/C (goat polyclonal #sc5216; Santa Cruz Biotechnology) were used as housekeeping controls for protein loading. Immunoblotted membranes were incubated with IRDye infrared-conjugated secondary antibody (LI-COR Bioscience, Lincoln, NE). Proteins of interest were detected by LI-COR imaging system. Densitometry was performed using ImageJ Software (NIH 1.45s) to quantify the level of these proteins (http://rsbweb.nih.gov/ij/).

**Statistical Analyses.** Data are presented as the mean ± S.E.M. for n independent experiments. Each experiment was repeated on a minimum of three separate occasions. All data were statistically analyzed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). One or two-way analysis of variance with repeated measures were used to analyze the data, in most cases, followed by Bonferroni’s post hoc tests to compare treatment groups. A P value of < 0.05 was considered statistically significant.

**Results**

*RvD2 Promotes MCF-7 but Not MDA-MB-231 Cell Proliferation.* RvD2 (1–100 nM) increased MCF-7 cell number after 48 hours. However, it had no detectable effect on MDA-MB-231 cell number (Fig. 1, A–C). Further investigations indicated that the increased cell number was more likely due to proliferation, as neither viability nor rates of apoptosis were affected by RvD2 treatment, as measured by flow cytometry (data not shown). Because MCF-7 cells are responsive to estrogen and are a well established in vitro model for ERα-positive breast cancer and MDA-MB-231, an in vitro model for triple-negative (ERα, PR, HER2-negative) breast cancer, this unexpected result triggered our investigation into the role of ER activation in the apparent proliferative effect of RvD2. Therefore, we cotreated MCF-7 cells with the estrogen receptor antagonist ICI 182,780, which significantly inhibited E₂- and RvD2-induced proliferation (Fig. 1D). This observation prompted us to further characterize the estrogenicity of RvD2-induced increases in cell number.

*RvD2 Proliferative Effect Is Prevented by the PI3K/Akt Inhibitor LY294002.* RvD2 induced mitogenesis was inhibited by the PI3K/Akt inhibitor, LY294002, but not by the
ERK1/2 inhibitors (PD98059 and UO126) or p38MAPK inhibitors (SB202190 and SB203580) (Fig. 2).

**RvD2 Enhances ERE Transcriptional Activity in a Panel of ERα-Positive Cancer Cell Lines.** Hormone-activated ER mediates the transcriptional activation of target genes by binding to the specific DNA sequence ERE within their promoters (Hall et al., 2001). RvD2 increased ERE transcriptional activity in a concentration-dependent manner in a number of ERα-positive breast tumor cell lines (MCF-7, T47D, BT474) and an ER-positive ovarian tumor cell line (SKOV3). Prior exposure to ICI 182,780 prevented this stimulation (Fig. 3).

**RvD2 Modulates Expression of Estrogen Responsive Genes.** Having demonstrated that RvD2 increases ERE-SEAP activity, we then ascertained whether it modulates the transcription of estrogen-responsive genes. Growth regulated by estrogen in breast cancer 1 (GREB1) and progesterone receptor are considered markers of estrogen responsiveness (Rae et al., 2005; Bonéy-Montoya et al., 2010). RvD2 significantly increased GREB1 and progesterone receptor mRNA levels in MCF-7 cells (Fig. 4, A and B). Furthermore, RvD2 upregulated cyclin D1 (CCND1) mRNA expression, one of the genes that estrogen induces in breast tumor cells to mediate its mitogenic effect (Fig. 4C) (Peurala et al., 2013). The PI3K/Akt inhibitor LY294002 attenuated both E2- and RvD2-induced CCND1 upregulation (Fig. 4D). On the other hand, RvD2 significantly reduced the expression of TGFβRII and TGFβ3 genes, which are associated with the suppression of breast cancer cell proliferation and known to be downregulated by estrogen (Fig. 4, E and F) (Arrick et al., 1990; Frasor et al., 2003). These effects were all blocked by the administration of ICI 182,780.

**RvD2 Does Not Compete with E2 for ER Binding.** Based on these observations, we performed competitive radioligand binding assays to assess the capacity of RvD2 to directly bind to the E2 binding site on ER. In both intact MCF-7 cells and cytosol, RvD2 did not displace the binding of [3H]E2 (Fig. 4, A and B). However, treating MCF-7 cells with RvD2 for 30 minutes before cytosolic extraction resulted in a significant reduction in [3H]E2 binding sites (Fig. 5, C and D).

**RvD2 Induces ERα Nuclear Localization.** We showed that prior exposure of MCF-7 cells to RvD2 resulted in a marked reduction in [3H]E2 binding sites in the cytosol. One explanation for this observation is that cytosolic ER has translocated to the nucleus. Moreover, steroid hormone receptors, including ERα, form stable complexes with the chromatin matrix in the nucleus on hormone stimulation (Leclercq et al., 2006). Therefore, we next investigated the mechanism of ERE activation in MCF-7 cells by establishing the levels and subcellular location of ERα in cells treated with RvD2 or E2 for 30 minutes. Total ERα protein level was not affected by RvD2 or

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**Fig. 4.** Effect of RvD2 on PGR (A), GREB1 (B), CCND1 (C and D), TGFβR2 (E), and TGFβ3 (F) mRNA levels normalized to concurrently measured 18S rRNA and referenced to vehicle-treated cells. MCF-7 cells were pretreated with 182,780 (A–C, E, and F) or LY294002 (D) for 30 minutes before incubation with E2 or RvD2 for another 4 to 24 hours. RNA was extracted and mRNA expression was measured by reverse-transcription polymerase chain reaction. Data represent means and the S.E.M. of n = 3–7. *P < 0.05; **P < 0.001; ***P < 0.0001 versus control. •P < 0.05; ••P < 0.001; •••P < 0.0001 versus E2 or RvD2 (1 μM). PGR, progesterone receptor; TGF, transforming growth factor.
E2 incubation in these cells (Fig. 6B). However, examination of ERα by immunofluorescence revealed that both E2 and RvD2 increased the nuclear levels of ERα (Fig. 6A). To further describe the impact of RvD2 on ERα nuclear localization, an immunoblot of nuclear and cytosolic extracts of cells incubated with E2 or RvD2 was undertaken. Immunoreactive-ERα levels increased in the nucleus after treatment with either E2 or RvD2 (Fig. 6, C–E).

Discussion

We sought to characterize the impact of RvD2 on breast tumor cell growth. Our findings suggest that RvD2 is estrogenic and supports ERα-positive cancer cell growth via ER signaling at concentrations that overlap with the physiologic concentrations of RvD2 in human circulation (Mas et al., 2012). RvD2-induced increases in MCF-7 cell number were most likely due to proliferation, as neither viability nor rates of apoptosis were affected by RvD2 treatment. We have also shown that the proliferative action of RvD2 was prevented by the ER antagonist ICI 182,780. Furthermore, RvD2 concentration dependently increased ERE-SEAP transcriptional activity in ER-positive breast tumor cell lines (MCF-7, T47D, BT474) and in an ovarian tumor cell line (SKOV3). The enhancement of ERE transactivation was shown to have pathophysiological relevance, as it was paralleled by upregulation of GREB1 and PGR mRNA levels, well characterized ERE-containing estrogen-inducible genes (Deschênes et al., 2007; Gohno et al., 2012). The specificity of this phenomenon was confirmed by the observation that the RvD2 precursor docosahexaenoic acid did not have any impact on MDA-MB-231 and MCF-7 cell proliferation or ERE transactivation (data not shown).

RvD2 increased CCND1 gene expression, a key player in the initiation of cell-cycle progression and a gene known to be induced by estrogens. In addition, CCND1 has recently been shown to be required for estrogen-dependent expression of a subset of genes involved in growth factor and cytokine signaling in vivo by facilitating the formation of ERα-coactivator complexes (Casimiro et al., 2013). Of note, CCND1 is overexpressed in approximately 30–50% of cancers and is considered the most frequently overexpressed gene in breast cancer. Its amplification is associated with poor prognosis (Casimiro et al., 2014).

Data obtained from the proliferation experiments suggest that RvD2 produces its proliferative effect through PI3K/Akt signaling, because its effects were abrogated by the PI3K/Akt inhibitor (LY294002). Moreover, we have demonstrated that both E2- and RvD2-induced upregulation of CCND1 were also inhibited by LY294002. It is well established that cell survival and cell proliferation are mediated predominantly through the PI3K/Akt and the ERK1/2 MAPK pathways. These kinases are often found to be highly deregulated in cancer, and their activation will ultimately result in upregulation of CCND1 expression (Castaneda et al., 2010; Vadlakonda et al., 2013). Moreover, they are also important for ER activity in some tumors because they phosphorylate and thereby activate both E2-free and E2-bound ER (Renoir et al., 2013). This ER phosphorylation augments the transcriptional activation potential of ER and enhances its effects on cell proliferation and survival.

Exposure of MCF-7 cells to RvD2 before the cytosolic extraction resulted in significant reduction in [3H]E2 binding sites in the cytosol as evident from the radioligand binding experiment (Fig. 5, C and D). Regardless of the extent to which the displacing concentration of E2 was back-titrated, the data clearly show that the displacement curve was not left shifted. There was an apparent loss of binding sites. That is to say, prior exposure to RvD2 decreased the apparent ER density in the cytosol. RvD2 enhancement of proteasomal...
degradation of ERα, a known characteristic of ERα ligand (Lee and Lee, 2012), can be excluded as an explanation of the loss of cytosol ERα, as ERα protein level (membrane + cytosol + nucleus) was not affected by RvD2 or E2 treatment (Fig. 6B). These observations led us to conclude that RvD2 caused translocation of ER from the cytosol to the nucleus. This possibility was confirmed by both immunofluorescence and immunoblotting studies, which indicated that RvD2 increased the nuclear localization of ER, which may explain the subsequent gene transcription. It is evident that the fraction of liganded ER involved in gene transactivation must be present in the nucleus. Moreover, it is established that activation of ER (e.g., by ligand binding) causes stabilization of ER interactions with the chromatin (Leclercq et al., 2006). Thus, increased ER nuclear localization is consistent with RvD2 estrogenic actions. However, our results do not exclude additional influences of RvD2 on the recruitment or derecruitment of ER coactivators or corepressors, respectively, or an influence on regulatory phosphorylation sites on ERα as underlying the estrogenic actions of RvD2.

Our observations parallel, to some extent, those in a recent report by Russell and colleagues in which they demonstrate an estrogenic profile for lipoxin A4 (LXA4) in human endometrial adenocarcinoma (Ishikawa) and endometrial epithelial (hTert EEC) cell lines (Russell et al., 2011). They suggest that LXA4 bioactivity involves direct binding to ER. However, competitive radioligand binding assays that we performed show clearly that RvD2 did not compete with [3H]E2 for ER binding, which excludes the possibility that RvD2 actions are mediated via binding to the orthosteric 17β-estradiol binding site. In addition, we observed that cotreatment with E2 and RvD2 did not inhibit the E2-induced increases in cell number or the expression of estrogen responsive genes (data not shown), in contrast to the antiestrogenic actions of LXA4 observed in endometrial epithelial cells. However, several important differences in the two studies could account for these distinct bases for estrogenic actions, including differences in ER population in endometrial versus breast epithelial and carcinoma cells and the interaction of ligand-receptor complexes with different coactivator/corepressor proteins. In addition to the ER itself and the genomic response element to which it binds, a number of other cellular adaptor proteins are involved in determining the response of individual cells to estrogenic stimuli. One important example of these coregulatory proteins is the steroid receptor coactivator-1 (SRC-1). It has been shown that its overexpression can potentiate the transcriptional activity of tamoxifen-activated estrogen receptor. On the other hand, overexpression of the corepressor silencing mediator of retinoic acid and thyroid hormone (SMRT) in target cells permitted tamoxifen-activated ER to manifest partial agonist activity, and overexpression of steroid receptor coactivator-1...
was unable to reverse this process (Smith et al., 1997). Collectively, this indicates that the relative expression of coactivators and corepressors can modulate ligand regulation of ER transcriptional activity and suggest that they contribute to the tissue-specific ability of certain compounds to activate or inhibit ER-mediated gene expression. Most importantly, although LXA₄ is thought to act through formyl peptide receptor 2, the receptor for RvD₂ remains unknown. Thus, there is no reason to expect these trihydroxy polyunsaturated fatty acids will behave concordantly.

Our results also exclude the possibility that RvD₂ functions as an allosteric modulator of ER because it did not alter [³H]E₂ affinity for ER binding. However, ER transcriptional activity does not necessarily entail direct ligand binding. Small hydrophobic signaling molecules (e.g., dopamine (Power et al., 1991; Ribly et al., 2000), growth factors (e.g., epidermal growth factor [EGF], IGF-1 [Ignar-Trowbridge et al., 1996; Marquez et al., 2001]), as well as components downstream of membrane receptors, such as second messengers (Zhang et al., 2013), have been shown to influence ER-mediated gene expression. These various molecules influence the phosphorylation status of the receptor through signal transduction pathways (for instance, phosphorylation of Ser118 by p44/42 MAPK when EGF receptor is activated by EGF) (Bunone et al., 1996). Transcriptional coregulators are also targets for these signal transduction pathways (Driggers and Segars, 2002). Evidence has been reported to support the idea that growth factor signaling acts in synergy with estradiol for optimal transcriptional effects (Marquez et al., 2001). Thus, RvD₂ may activate signaling cascades that lead to post-translational modifications of ERα or its coactivators or corepressors.

Another possibility which could explain RvD₂ estrogenicity, and we have not explored in this study the involvement of the plasma membrane–associated estrogen binding protein, GPER1, previously named GPR30. GPER1 is a membrane-spanning protein and a member of the G protein–coupled receptor family. GPER1 has been proposed to mediate both genomic and nongenomic actions of E₂. It has been demonstrated that GPER1 can promote the progress of ER-positive tumors through MAPK signaling pathway (Wang et al., 2010). However, it has also been shown that GPER1 is proapoptotic in ER-positive breast cancer cells, and its expression correlates with increased distant disease-free survival of ER-positive breast cancer patients (Broselid et al., 2013). We have previously made extensive efforts to establish the contribution of this receptor in ER-positive breast cancer cell lines using the commercially available synthetic GPER1 agonist, G1, and failed to observe any of the known biologic effects of estrogen mediated through this receptor (Tan, Stewart, and Ziogas, unpublished observations). Taken together with the clear inhibitory effect of ICI 182,780, we conclude that it is unlikely that signaling through the GPER1 could explain the observed RvD₂ effects.

This study defines a potential role for RvD₂ as a modulator of ERα signaling in ER-positive breast cancer cells with effects on proliferation and ER-dependent transcription. RvD₂ effects did not involve direct binding to ER, nor did it alter binding of E₂ to the ER. RvD₂ effects appear to be as a result of activation of the PI3K/Akt signaling pathway with subsequent upregulation of cyclin D₁ expression (Fig. 7). Further investigations are necessary to fully characterize RvD₂-elicted effects in physiologic and pathologic conditions of the mammary glands.

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Participated in research design: Stewart, Al-Zaubai, Johnstone, Rizzacasa.
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