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Progestin modulates the lipid profile and sensitivity of breast cancer cells to docetaxel

Isabel R. Schlaepfer^{*}, Carolyn A. Hitz^{*}, Miguel A. Gijón[&], Bryan C. Bergman^{*}, Robert H. Eckel^{*}, and Britta M. Jacobsen^{*}

^{*}Department of Medicine, Division of Endocrinology, Metabolism, and Diabetes, University of Colorado Anschutz Medical Campus

[&]Department of Pharmacology, University of Colorado Anschutz Medical Campus

Abstract

Progestins induce lipid accumulation in progesterone receptor (PR)-positive breast cancer cells. We speculated that progestin-induced alterations in lipid biology confer resistance to chemotherapy. To examine the biology of lipid loaded breast cancer cells, we used a model of progestin-induced lipid synthesis. T47D (PR-*positive*) and MDA-MB-231 (PR-*negative*) cell lines were used to study progestin response. Oil red O staining of T47D cells treated with progestin showed lipid droplet formation was PR dependent, glucose dependent and reduced sensitivity to docetaxel. This protection was not observed in PR-*negative* MDA-MB-231 cells. Progestin treatment induced stearoyl CoA desaturase-1 (SCD-1) enzyme expression and chemical inhibition of SCD-1 diminished lipid droplets and cell viability, suggesting the importance of lipid stores in cancer cell survival. Gas chromatography/mass spectroscopy analysis of phospholipids from progestin-treated T47D cells revealed an increase in unsaturated fatty acids, with oleic acid as most abundant. Cells surviving docetaxel treatment also contained more oleic acid in phospholipids, suggesting altered membrane fluidity as a potential mechanism of chemoresistance mediated in part by SCD-1. Lastly, intact docetaxel molecules were present within progestin induced lipid droplets, suggesting a protective quenching effect of intracellular lipid droplets. Our studies suggest the metabolic adaptations produced by progestin provide novel metabolic targets for future combinatorial therapies for progestin-responsive breast cancers.

Keywords

Progestin; metabolism; breast cancer; SCD-1; lipid droplets; docetaxel

1. Introduction

Although the Warburg effect has been recognized for over fifty years (WARBURG 1956), less understood are alterations in lipid metabolism and the rapid rates of *de novo* fatty acid

Correspondence: Britta M. Jacobsen, University of Colorado Anschutz Medical Campus, Department of Medicine, Division of Endocrinology, Metabolism and Diabetes, MS 8106, 12801 E. 17th Ave., Aurora, CO 80045, Tel: 303 724-3942, Fax: 303 724-3920, Britta.jacobsen@ucdenver.edu.

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(Menendez et al., 2007). Another enzyme involved in lipid synthesis is stearoyl-CoA desaturase (SCD-1), which generates monounsaturated palmitoleic and oleic acids which become part of the phospholipid bilayer and/or components of lipid droplets. Inhibition of SCD-1 in breast cancer cells blocks lipid synthesis, decreases growth and viability, making it an ideal target for therapeutic intervention (Scaglia et al., 2009). However nothing is known about the mechanisms of SCD-1 action in progestin-sensitive breast cancers.

The mechanism of progesterone-mediated lipogenesis in breast cancer has not been explored in depth, especially in the context of metabolism and chemotherapeutic response. In these studies we investigated the role of the progestin medroxyprogesterone acetate (MPA) on lipid accumulation and chemotherapeutic response of two breast cancer cell lines that differ in their PR content. We found that progestins promote PR-mediated metabolic changes that favor chemotherapy survival in breast cancer cells by three mechanisms: 1) inducing *de novo* lipid synthesis using glucose from the growth medium, 2) modifying the phospholipid composition of membranes (greater unsaturated fatty acid content) and 3) sequestering docetaxel within enlarged lipid droplets. Since progestins are widely used in hormone replacement therapy and oral contraception (Ito et al., 2010), understanding their mechanisms of action at the cellular and metabolic levels are of critical importance and may provide novel targets for metabolic cancer therapies.

2. Methods

2.1 Cell culture and drug treatments

T47D and MDA-MB-231 (gifts from Kathryn Horwitz, University of Colorado) were authenticated by Single Tandem Repeat analysis (STR; University of Colorado Cancer Center) and were passaged in MEM (Invitrogen) containing 5% Fetal Bovine Serum supplemented with amino acids and Insulin (Hyclone). Drug treatments were done in phenol-red free 5% dextran-coated charcoal serum (DCC) to eliminate serum steroids. Medroxyprogesterone acetate (MPA; Sigma) was solubilized in ethanol and used at 10nM concentration for all experiments. ZK98,299 (Schering AG) was dissolved in ethanol and used at 100nM concentration. Docetaxel (LKT Labs) was solubilized in ethanol and used at 100nM concentration. SCD-1 inhibitor (#1716-1; 4-(2-Chlorophenoxy)-N-(3-(3-methylcarbamoyl)phenyl)piperidine-1-carboxamide) was purchased from BioVision (Mountain View, CA), dissolved in DMSO and used at 50nM. Glucose-depleted media was obtained from Sigma-Aldrich.

2.2 Analyses for lipid content and proliferation

Breast Cancer cells (1×10^5 T47D; 5×10^4 MDA-MB-231) were plated on coverslips placed in 6-well plates and treated with ethanol or MPA (10nM). Cells were fixed with 4% Paraformaldehyde for 10 min followed by 4 min incubation with 50% ethanol at room temperature. Cover-slips were kept at 4°C in PBS until use. Oil red O staining was performed on the fixed coverslips according to the standard protocol (Koopman et al., 2001). Cells were imaged using a Nikon Eclipse Ti microscope mounted with a Nikon DS-Qi1Mc digital camera with Nikon Instruments Software (NIS) Elements software. Ten fields of each condition were used for the quantification of the Oil red O staining, using the NIH

ImageJ software freely available on the web. Cell proliferation was performed using the Beckman Coulter Vi-Cell Automated Cell Viability Analyzer, which automates the widely accepted trypan blue cell exclusion method. For Vi-Cell analysis, cell densities were plated as above in 12-well dishes and treated with ethanol or MPA for 4 days in phenol-free 5% DCC serum-containing media. Cells treated with docetaxel were incubated for 2 additional days.

2.3 Immunoblots

For electrophoresis, 80µg of whole cell extracts were run on a 7.5% SDS-PAGE gel and transferred to nitrocellulose (Invitrogen). After blocking with 5% non-fat dry milk in PBS containing 0.1% Tween 20 (PBST), blots were incubated overnight with the primary antibody to PR (1:2000 mouse anti-PgR1294: Dako,); SCD-1 (1:500, Santa Cruz) or beta-actin (1:1000 rabbit, Cell Signaling). Blots were washed 3 times in PBST and followed by 1 hour incubation with corresponding secondary antibodies conjugated to horseradish peroxidase (Invitrogen). Band signals were visualized with chemiluminescence ECL substrate (Pierce) and measured densitometrically using the AlphaEaseFC software (Alpha Innotech).

2.4 Glucose uptake

Basal and insulin-mediated glucose uptakes were determined as previously reported for human cells *in vitro* (Sarabia et al., 1992). Briefly, T47D cells (1×10^5) in 6-well plates were incubated in Krebs-Ringer bicarbonate-HEPES (KRBH) buffer (130 mM NaCl, 5mM KCl, 1.3 mM CaCl₂, 1.3 mM MgSO₄, 25 mM HEPES, pH 7.4) containing 1% fatty acid-free bovine serum albumin (Sigma-Aldrich), 2-deoxyglucose (0.5 mM; Sigma-Aldrich), and [1,2-³H]2-deoxy-Dglucose (1uCi/mL; GE Healthcare) for 20 min at 37°C. For insulin stimulation, a set of cells were incubated with 100 nM insulin for 20 minutes. The cells were washed with ice-cold KRBH buffer, harvested in 800ul of lysis buffer (Pierce Biotechnology), and then added to scintillation vials containing 5 ml of scintillation fluid. Radioactive counts were determined with a scintillation counter (LS6500; Beckman Coulter). Counts were converted to moles of glucose taken-up and normalized to the protein concentration of the lysates. To account for deoxyglucose uptake not mediated by transporters, counts were also measured in the presence of 20 µM cytochalasin B (Sigma-Aldrich) to obtain glucose transporter-mediated uptake.

2.3 Lipid analysis: Gas Chromatography-Mass Spectroscopy

T47D cells were grown in 60mm plates and treated with ethanol or MPA for 6 days, with or without docetaxel for the last 48h. Cells were collected in 2 mls of PBS and an aliquot of 100µL was used for cell count using a hemocytometer for standardization. Lipids were extracted using the Bligh and Dyer method (BLIGH et al., 1959). Samples were shaken on a rotational mixer for 1.5 hours at 4°C, and then spun at 3,000 rpm for 15' to separate phases. The organic bottom layer was dried down under N₂ at 40°C, resuspended in chloroform, and added to aminopropyl solid phase extraction (SPE) columns (Supelclean LC-NH₂, 3ml, Supelco Analytical). Phospholipids were isolated using SPE based on the methods originally described by Kaluzny (Kaluzny et al., 1985). The FFA fraction was methylated by adding

0.5 ml 2% sulfuric acid, capping, and heating at 100°C for 1.5 hours. Phospholipid fractions were converted to fatty acid methyl esters (FAME) by transmethylation using sodium methoxide. Concentration and composition analysis were performed on an HP 6890 gas chromatography system with a 30m DB-23 capillary column, connected to a HP 5973 mass spectrometer. Peak identities were determined by retention time and mass spectra compared to standards of known composition.

2.4 Lipid droplet isolation

T47D cells were grown in T-175 flasks and treated with MPA for 6 days with or without docetaxel as described above. Lipid droplets were isolated from a sucrose gradient as described (Taylor et al., 1997), with minor modifications: cell homogenates in 0.5M sucrose were cleared by a slow spin for 10min at 1300×g and the post-nuclear supernatants were loaded into SW40 ultra-clear tubes and centrifuged at 28,000rpm (100,000×g) for 1 hour. After centrifugation, the thin opaque lipid droplet layer was collected, transferred to a glass collection tube and kept at -80°C for lipid extraction and liquid chromatography-tandem mass spectrometry (LCMS/MS).

2.5 Liquid chromatography-tandem mass spectrometry (LCMS/MS) for docetaxel

An aliquot of the lipid droplet preparation was mixed with 1 volume of ice-cold methanol, and paclitaxel (2ng, Sigma) was added to every sample as internal standard. Samples were diluted with water to a final methanol concentration under 15% and then applied onto SPE cartridges (Strata-X, 33 µm Polymeric Reversed Phase, 60 mg; Phenomenex, Torrance, CA). Cartridges were washed with 4 ml water and lipids were eluted with 1 ml methanol. The eluate was dried down and solubilized in a mixture of 40 µl HPLC solvent A (8.3 mM acetic acid, buffered to pH 5.7 with ammonium hydroxide) and 20 µl HPLC solvent B (acetonitrile/methanol, 65:35, v/v). An aliquot of each sample (25 µl) was injected into a C18 HPLC column (Ascentis 5 µm, 150 × 2 mm; Sigma) and eluted at a flow rate of 200 µl/min with a linear gradient from 45% to 98% HPLC solvent B, as follows: 45% B for 5 min, increased to 98% B in 14 min, held at 98% for 6 min, decreased to 45% B in 1 min, and re-equilibrated at 45% B for 4 min. The HPLC system was directly interfaced into the electrospray source of a triple quadrupole mass spectrometer (API 3000; PE-Sciex, Thornhill, Ontario, Canada) for mass spectrometric analysis in the positive ion mode using multiple reaction monitoring of the specific mass-to-charge (m/z) transitions (parent-ion → product-ion) 808 → 527 (docetaxel) and 854 → 286 (paclitaxel), which have been previously used to identify docetaxel (Corona et al., 2011). Analysis was repeated with samples from three different experiments.

2.6 Statistics

All values are given as means plus standard deviations (SD). Differences in parameters between treatments were assessed by ANOVA tests followed by Tukey post-hoc analysis. A p value of 0.05 was accepted as statistically significant.

3. Results

3.1 Progesterin-induced lipid accumulation is associated with chemoresistance and mediated by PR

The luminal PR-positive breast cancer cell line T47D accumulates lipid when treated with progesterin (Chalbos et al., 1987; Horwitz et al., 1985). Basal-like MDA-MB-231 PR-negative breast cancer cells were also used to examine the lipid biology of PR+ versus PR- breast cancer cells. We have examined the lipid content of the two cell lines, untreated or treated with the progesterin MPA, using Oil red O (ORO) staining. Lipid staining was absent in MDA-MB-231 cells (not shown) but visible in the T47D cells (Fig. 1A). We also examined whether lipid droplet accumulation was affected by the chemotherapeutic agent, docetaxel. Docetaxel is a commonly used chemotherapeutic agent to treat advanced breast cancer (Lyseng-Williamson et al., 2005). Cells were treated with MPA or vehicle for 4 days to induce lipid accumulation followed by 2 days with or without docetaxel (with continuous MPA or vehicle treatment) and lipid content was assessed by ORO staining. A second set of cells grown in parallel was used to assay cell viability by trypan blue exclusion. Fig. 1A shows representative ORO photos of the T47D cells in the absence or presence of MPA, treated with or without docetaxel. MPA alone induces lipid accumulation as previously reported (2nd panel, left), and the combination of MPA+docetaxel also showed increased accumulation of lipid droplets (2nd panel, right). Interestingly, viable cells had lipid accumulation in the cytoplasm (Fig. 1A, 2nd panel, right, green arrow), while cells with apoptotic features (condensed DNA and/or fragmented nuclei) did not contain lipid droplets (black arrows).

To examine whether the lipid accumulation observed in the cells was PR dependent, the pure PR antagonist ZK98,299 (Classen et al., 1993) was used to block the effect of MPA on PR (Fig. 1A, 3rd and 4th panels). ZK98,299 was added for the entire incubation period with or without MPA and with or without docetaxel. ZK98,299 blocked the lipogenic effect mediated by MPA demonstrating that PR is necessary for progesterin-mediated lipid accumulation in T47D cells. Quantification of lipid droplets shows MPA increased lipid formation ~4 fold over control in the PR+ T47D cells ($p = 0.04$) (Fig. 1B). The combination of docetaxel+MPA increased lipid accumulation 6.5 fold versus docetaxel alone ($p = 0.001$). We also examined two other PR+ breast cancer cell lines, BT-474 and ZR-75-1, and observed the same trend of MPA and MPA+docetaxel induced lipid accumulation (Supplementary Fig. 1A). We also examined PR levels in the three cell lines; T47D, BT-474 and ZR-75-1 and all express both isoforms of PR (PRA and PRB) (Supplementary Fig. 1B). The two PR isoforms can have differential effects on breast cancer biology (reviewed in Scarpin et al., 2009). To determine the contribution of each PR isoform to MPA induced lipid accumulation, lipid droplets were examined in T47D cell sublines that express no PR (T47D-Y cells), PRA only (T47D-YA cells), or PRB only (T47D-YB cells) (Sartorius et al., 1994). Lipid droplet accumulation was observed solely in breast cancer cells co-expressing PRA and PRB (Supplementary Fig. 2) but not in cells expressing only PRA, only PRB or no PR. Similarly, the PR-negative MDA-MB-231 cells do not accumulate lipid in response to MPA, docetaxel or the combination (Fig. 1B), underscoring the necessity of PR in mediating the response to MPA-induced lipid accumulation. Next we examined whether the increased

lipid accumulation by MPA was associated with cell survival against docetaxel induced apoptosis, using trypan blue exclusion analysis. Fig. 1C shows the number of surviving cells with and without MPA and/or docetaxel treatment. MPA did not alter viability of MDA-MB-231 cells in the absence of docetaxel, however, docetaxel caused an 80% decrease in viable cells regardless of MPA treatment ($p<0.01$). This sensitivity is likely due to the high proliferation rate of MDA-MB-231 cells. After 6 days of MPA treatment, the number of T47D cells was slightly decreased versus untreated cells (1.25 fold) ($p=0.1$). Docetaxel decreased the number of T47D cells 1.7-fold in the absence of MPA ($p=0.02$). However, in the presence of MPA, docetaxel did not decrease the number of T47D cells significantly (1.4 fold, Fig. 1C), illustrating a more docetaxel resistant phenotype of progestin treated cells. In fact, microscopic examination of the MPA+docetaxel treated T47D cells showed lipid accumulation in the cytoplasm of viable cells but not in apoptotic cells (Fig. 1A). Furthermore, T47D cells that survived treatment with docetaxel+MPA contained more lipid than cells treated with MPA alone (Fig. 1B). Thus only viable T47D cells accumulate lipid droplets in response to docetaxel therapy and progestin decreases the sensitivity of PR+ cells to docetaxel induced apoptosis.

Progestins induce a biphasic change in the cell cycle of PR+ breast cancer cells, first inducing proliferation, then subsequent growth inhibition in G1/G0 for up to 96h (Musgrove et al., 1991). To determine whether T47D cells arrest in G1/G0 after 6 days of MPA-treatment therefore making them less sensitive to docetaxel, we performed flow-cytometry analysis (Supplementary Fig. 3A). MPA decreased the number of cells in G1/G0 and increased the percentage of cells in the S-phase of the cycle (8%, $p=0.012$). MPA also decreased the number of cells in G1/G0 upon docetaxel treatment with a concomitant increase in cells in G2M. We also examined cells for proliferation by BrdU incorporation. BrdU labeling increased in MPA-treated cells with or without docetaxel versus vehicle. Furthermore, BrdU accumulation was only observed in MPA treated cells containing lipid droplets with or without docetaxel, but in the absence of MPA none of the proliferating cells contained lipid droplets (Supplementary Fig. 3B and Fig. 3C). These data confirm MPA induces proliferation and show that lipid-loaded MPA-treated cells are not protected from docetaxel induced apoptosis by cell cycle arrest (Supplementary Fig. 3B and 3C). These data are consistent with previous reports that long term progestin treatment increases proliferation and antiapoptotic proteins in T47D cells (Moore et al., 2000).

3.2 Lipid droplet formation requires glucose; progestin increases glucose uptake

Glucose is a common precursor for *de novo* lipid formation. To investigate whether glucose in the culture media was required for MPA-induced lipid droplet formation, T47D cells were cultured in the presence or absence of glucose with or without MPA for 4 days (Fig. 2A). In contrast to the abundant lipid droplets induced by MPA in glucose containing media, glucose starved cells were unable to produce lipid droplets, illustrating that glucose uptake is absolutely required for lipid droplet formation in T47D cells (Fig. 2A). Because 1) lipogenesis increases in progestin treated T47D cells (Fig. 1), 2) glucose is required for MPA-induced lipid accumulation and 3) the media in which the cells are cultured is rich in glucose (25 mM), we speculated that MPA increased glucose uptake in T47D cells as a substrate for *de novo* lipid synthesis, which is mediated by the FASN enzyme from glucose

(see Fig. 4A). Progestins increase the levels of insulin receptors in the plasma membrane and their effect could be associated with lipid accumulation (Horwitz et al., 1985), so we examined the effect of insulin-mediated glucose uptake in MPA-treated T47D cells. Fig. 2B shows the basal and insulin stimulated level of glucose uptake of T47D cells treated with or without MPA with or without docetaxel as measured by H³-2deoxy-labeled glucose. The microtubule inhibitor, Cytochalasin B (Cyto-B) was used to assess passive glucose uptake not mediated by transporters. Glucose uptake is low in cells treated with Cyto-B, however, MPA slightly stimulates glucose uptake 1.6-fold versus vehicle. Glucose uptake is 6 fold higher in the absence of Cyto-B (basal), and MPA stimulates glucose uptake 1.4-fold ($p=0.054$) compared to vehicle alone. Interestingly, insulin stimulation does not significantly increase glucose uptake above the basal levels in the absence of MPA, but MPA+insulin increases glucose uptake (1.8 fold, $p<0.001$), which may be mediated by insulin-sensitive transporters like GLUT4. Furthermore, when comparing basal MPA uptake to insulin+MPA glucose uptake, a significant increase was observed in the absence of docetaxel (1.4 fold, $p<0.01$), suggesting a possible synergistic effect of MPA and insulin in T47D cells. Of note, progestin-induced glucose uptake is predominantly due to glucose transporters at the plasma membrane as previously reported (Medina et al., 2002).

Docetaxel treatment did not inhibit MPA induced glucose uptake. MPA treated cells showed a slight increase in passive glucose uptake (Cyto-B) and a significant 2.0 fold ($p = 0.02$) increase in basal glucose uptake. Insulin did not significantly further increase glucose uptake in the absence or presence of MPA. Thus, the majority of glucose uptake from the media is mediated by glucose transporters and glucose is the substrate used to generate the lipids observed in MPA-treated T47D cells, irrespective of docetaxel treatment.

3.3 SCD-1 expression is induced by progestin and it is required for lipid synthesis and cell growth

One mechanism of increased *de novo* fatty-acid synthesis is the increased expression/activity of lipogenic enzymes. Among them, fatty acid synthase (FASN) is expressed in many cancers and is also induced by progestins (reviewed in (Mashima et al., 2009)). Although FASN was more abundant in T47D than MDA-MB-231 cells, we did not observe induction of FASN upon 4 or 6 days of MPA (Supplementary Fig. 4A and Fig. 4B), implying that MPA induction of FASN occurs earlier than 4 days. Another enzyme, SCD-1, is essential for the processing/storage of lipids (Scaglia et al., 2009). SCD-1 converts fatty acids from saturated to unsaturated for use in phospholipid bilayer and/or storage in lipid droplets. Next we examined whether MPA affects SCD-1 expression. Quantitative RT-PCR showed that MPA strongly increased SCD-1 mRNA (Supplementary Fig. 5). MPA induced SCD-1 protein ~2-fold in the absence or presence of docetaxel, although SCD-1 expression was significantly reduced by docetaxel (Fig. 3A), probably due to the toxic effects of docetaxel.

In order to examine the effects of blocking SCD-1 on MPA-mediated lipogenesis and cell survival, we used a chemical inhibitor of SCD-1 activity (BioVision, #1716). Cells treated with MPA or vehicle with or without docetaxel were exposed to the SCD-1 inhibitor and stained for lipid droplets (Fig. 3B) and the number of surviving cells was quantified (Fig.

3C). MPA increased lipid droplet formation ~5 fold, (Fig. 3B). In the presence of MPA +docetaxel the lipid droplets increased 6.5 fold over docetaxel alone as we observed previously. Addition of the SCD-1 inhibitor in the absence of progestin dramatically reduced basal lipid droplet formation ~10 fold (Fig. 3B). While MPA treatment slightly increased lipid droplet formation, it was ~10-fold less than in cells treated with MPA or MPA +docetaxel only (Fig. 3B). The combination of the SCD-1 inhibitor+docetaxel reduced basal lipid formation by 50% and progestin slightly increased lipid droplet formation to approximately baseline levels, implying that progestins regulate other pathways involved in the formation of lipid droplets. Next we examined cell viability with MPA, docetaxel and the SCD-1 inhibitor (Fig. 3C). As observed before, progestin priming followed by docetaxel treatment caused a less dramatic decrease in cell number (1.4 fold) versus the vehicle treated group. Treatment with the SCD-1 inhibitor decreased the number of cells 1.6 fold ($p=0.005$). Addition of MPA to the SCD-1 inhibitor further decreased viability 1.4 fold versus the SCD-1 inhibitor alone and 1.8 fold versus MPA alone ($p=0.006$). The combination of docetaxel+SCD-1 inhibitor was more toxic; the number of cells decreased 2.6 fold versus no treatment ($p<0.001$) and cell numbers decreased 1.5 fold versus either docetaxel or the SCD-1 inhibitor alone. While addition of MPA to the combination of docetaxel and the SCD-1 inhibitor caused a further decrease in viability, it was not statistically significant. These results suggest that SCD-1 is required for the lipid phenotype mediated by MPA and its inhibition is toxic to cancer cells. The SCD-1 inhibitor also affects cell viability in the absence of MPA (with few lipid droplets) suggesting that other functions of SCD-1 are important for cancer cell viability.

3.4 Progestin and docetaxel alter the phospholipid and triglyceride profile of T47D cells

Since SCD-1 expression is increased by MPA and it plays a role in lipid droplet formation, we examined other functions of SCD-1 in progestin treated T47D cells. Fig. 4A shows a diagram of function of the SCD-1 enzyme in lipid metabolism from glucose carbons. SCD-1 catalyzes the synthesis of monounsaturated fatty acids (palmitoleic and oleic) from saturated fatty acids (palmitic and stearic acids). All of these fatty acids contribute to the generation of storage and structural complex lipids like phospholipids (membranes) and triglycerides (lipid droplets). Therefore, we examined the fatty acid content of T47D phospholipids and lipid droplets after MPA and/or docetaxel treatments, using Gas Chromatography-Mass Spectroscopy (GCMS) analysis. Oleate (unsaturated) and palmitate (saturated) are the most common fatty acids circulating in blood, are abundant in cell membranes, and their levels are associated with breast cancer cell survival (Hardy et al., 2005; Przybytkowski et al., 2007). Table 1 shows the percentage of saturated lipids in the phospholipids (PL) and lipid droplets (LD) of T47D cells following MPA and/or docetaxel treatments. The majority of the saturated lipid (>74%) is found in the lipid droplets. However, we only observed significant changes among groups in the PL fraction (ANOVA, $p=0.003$). MPA decreased PL saturation 34% ($p=0.004$) when compared to vehicle treatment ($p=0.004$). Interestingly, docetaxel alone caused a similar decrease (23%) versus vehicle ($p=0.015$). MPA was not significantly different from MPA+docetaxel treatments. Within lipid droplets (LD) saturation content was slightly increased (~9%) upon MPA and/or docetaxel treatment but differences among treatments were not significant (Table 1).

To determine the major species of fatty acids that compose the phospholipids, we examined all fatty acid species. The four most common fatty acids in the T47D cells treated with or without MPA, with or without docetaxel were palmitic (saturated), palmitoleic (unsaturated), stearic (saturated) and oleic acids (unsaturated) (Fig. 4B). Importantly, only surviving cells were analyzed. We did not detect any arachidonic acid in the samples, which reflects the absence of linoleic acid, the essential fatty acid precursor obtained from the diet which is not observed in cells grown *in vitro*.

Palmitic acid and oleic acid are the predominant fatty acids of T47D cell membranes (Fig. 4B), and they are also abundant in aggressive cancer cells (Nomura et al., 2010). MPA increased oleic acid content (1.7-fold, $p=0.001$) and concomitantly decreased palmitic acid (1.5-fold, $p=0.002$) within phospholipids. Similarly, docetaxel increased oleic acid (1.5-fold, $p=0.002$) with a concomitant decrease in palmitic acid (1.3-fold, $p=0.005$) in phospholipids and this effect was not enhanced by MPA. Importantly, the altered ratio of oleic:palmitic acid was significantly different in cells treated with MPA alone versus docetaxel alone. Other saturated fatty acids (palmitoleic and stearic) were minor components of the phospholipid fraction but also decreased with MPA, docetaxel and MPA+docetaxel. Palmitoleic acid may be toxic as it is associated with tumor cell death in rodents (Ito et al., 1982), which may explain why cells treated with MPA, docetaxel or both decrease the palmitoleic acid content at cell membranes ($p<0.001$). Desaturation of stearic acid generates oleic acid, which is increased in MPA-treated and docetaxel-surviving cells, suggesting that the observed stearic acid decrease is due to desaturation to increase oleic acid. Interestingly, unbalanced levels of stearic (low) and oleic acid (high) levels are associated with breast cancer incidence in premenopausal women (Chajes et al., 1999). These data indicate that an altered ratio of fatty acids is a potential chemoresistance mechanism in breast cancer cells.

Figure 4C shows the predominant fatty acid within T47D lipid droplets was palmitic acid but we did not observe significant differences among treatment groups in the palmitic acid content of the lipid droplets (ANOVA, $p=0.13$). However, MPA showed a trend to decrease Palmitic acid ~23%, and docetaxel modestly decreased it (10%). Treatment with MPA +docetaxel caused a similar decrease in palmitic acid as MPA alone. No significant changes in palmitoleic acid were observed. Stearic acid was significantly increased with docetaxel alone (2.6 fold, $p=0.038$) and the combination of MPA+docetaxel ($p=0.03$) did not further affect this increase. MPA strongly decreased oleic acid (1.8 fold, $p=0.009$) in lipid droplets and the combination of MPA+docetaxel caused the same decrease in oleic acid as MPA alone ($p=0.009$). These studies suggest a reciprocal lipid profile between membranes and lipid droplets.

3.5 Intact docetaxel is sequestered in lipid droplets of progestin-treated cells

Because docetaxel is a lipid soluble drug that is found in lipoproteins within the plasma of cancer patients (Urien et al., 1996), we hypothesized that docetaxel could be sequestered in the MPA-induced lipid droplets of breast cancer cells. Thus, we isolated lipid droplets from T47D cells treated with MPA with or without docetaxel to examine them for intact docetaxel molecules. We used paclitaxel (analog of docetaxel) to serve as a reference peak for the analysis. The isolated lipid droplets were spiked with 2 ng of paclitaxel and then

subjected to lipid extraction and LCMS/MS. Figure 5 shows the ion intensity peaks for paclitaxel and docetaxel. The upper panel shows analysis of lipids isolated from MPA-only treated cells; docetaxel is not detected. The lower panel shows the lipid analysis of MPA +docetaxel treated cells and shows a docetaxel peak that is slightly resolved chromatographically from the paclitaxel internal standard. This intensity peak corresponds to intact docetaxel molecules (m/z 808). Since docetaxel works by binding microtubules in the cytoplasm, we also analyzed the aqueous phase from the lipid extraction, but we did not detect any docetaxel peaks (data not shown), suggesting that docetaxel molecules are strongly associated with intracellular lipid droplets.

4. Discussion

The response of breast cancer cells to progestins is both proliferative and inhibitory, depending on the culture conditions (Groshong et al., 1997; Lin et al., 1999; Moore et al., 2006; Musgrove et al., 1991). However, cancer cells that adopt a lipid-loaded state may be able to survive most chemotherapeutic treatments. This lipid-based resistance could be one mechanism by which tumors recur after completion of chemotherapy regimens (Liu et al., 2008). Although other studies have investigated the role of lipid enzymes in the resistance of cancer cells to chemotherapy (Menendez et al., 2005), studies examining viability and lipid content at the cellular level are lacking.

In our breast cancer model, the amount of progestin-induced lipid formation correlates with 1) the presence of PR, 2) increased glucose uptake, 3) the activity of SCD-1 lipogenic enzyme and 4) protection from docetaxel induced apoptosis. Importantly, PR+ cells that survived docetaxel treatment contained lipid droplets and appeared healthy. In contrast the PR-negative MDA-MB-231 cell line lacked lipid droplets and were sensitive to docetaxel. While this implies lipid droplets play a role in chemoresistance, the underlying mechanism(s) are unknown.

We speculate that the lipophilic nature of taxanes (like docetaxel) provides PR+ breast cancer cells a way to sequester intact taxane molecules in the lipid droplets and away from microtubules, on which docetaxel exerts its anti-tumoral actions. Supporting this speculation is the fact that docetaxel circulating in the plasma binds to the surface of plasma lipoproteins (circulating lipid droplets) with high affinity (Urien et al., 1996). Additionally, our results show that lipid droplets of MPA-treated T47D cells contain intact docetaxel molecules, suggesting that cancer cells that accumulate lipid are able to sequester active taxane molecules and favor cancer cells survival. These results have important clinical implications since combinatorial therapies that target lipid synthesis and growth may be more effective for steroid responsive tumors which are known to accumulate lipid, like breast and prostate cancers.

The lipogenic protective effect we observe in T47D cells may not be exclusive for taxanes. Cisplatin-resistant ovarian cancer cells are highly dependent on glucose uptake and lipid synthesis for survival, suggesting that a lipogenic phenotype is also a mechanism to escape cisplatin-induced cell death (Montopoli et al., 2011). If lipid loading in cancer cells is one mechanism promoting cancer cell survival, the positive effect of progestins on lipid

accumulation in breast cancer cells may help explain increased cancer mortality in individuals who received hormone replacement therapy (Chlebowski et al., 2010).

Identification of the lipid species in progestin-stimulated lipogenesis is also important for elucidating putative mechanisms of cancer cell survival. In breast cancer cells the saturated fatty acid palmitate increases apoptosis, while the unsaturated fatty acid oleic acid increases proliferation and protects against palmitate-mediated apoptosis (Hardy et al., 2000). Fatty acids produced endogenously by cancer cells, as opposed to exogenous lipids, are also associated with a malignant and aggressive phenotype (Nomura et al., 2010). Analysis of phospholipids in T47D cells treated with MPA, docetaxel or both revealed a decrease in the saturation of fatty acids versus controls, a result most likely due to increased expression/activity of the SCD-1 desaturase enzyme by MPA. The mechanism(s) of MPA mediated regulation of SCD-1 expression is currently unknown. Taken together, the MPA induction of SCD-1 expression and the decreased survival of cells exposed to MPA+SCD-1 inhibitor strongly suggest the effects of MPA are mediated in part by SCD-1 activity. SCD-1 is not an early response gene as it is not up-regulated by 6 hours of MPA treatment in T47D cells (Ghatge et al., 2005) however, SCD-1 is clearly up-regulated by MPA after 6 days of treatment. This implies that progestin mediated regulation of SCD-1 is indirect however, the underlying mechanism is unknown.

Further analysis of cellular lipids confirmed that unsaturated oleic acid (product of SCD-1 activity) as the most abundant fatty acid in phospholipids. Because fatty acid composition was analyzed *only* in cells surviving docetaxel treatment, this suggests increasing the oleic acid content of the cancer membranes alters its composition and enhances cell survival, as others have also suggested (Baritaki et al., 2007). Docetaxel treatment also increases polyunsaturated fatty acids in MCF7 breast cancer cells (Bayet-Robert et al., 2009), suggesting membrane fluidity as a survival mechanism not unique to T47D cells. Examination of saturation content between phospholipids and lipid droplets also suggests reciprocal lipid content between membrane and storage lipid droplets. We speculate that shunting palmitate to the lipid droplets is a protective mechanism of lipogenic cancer cells, so excess palmitate does not accumulate in cell membranes. The possibility that an MPA induced increase in the saturation content of lipid droplets (more palmitate/less oleic) favors the sequestration of docetaxel in lipid droplets is an intriguing, untested idea.

Abundant glucose provides chemoresistance in T47D cells *via* FASN activity (Zeng et al., 2010). In cancer cells, progesterone increases glucose transporters (GLUT-1/3) and consequently glucose uptake, favoring cancer progression and increasing breast cancer risk (Medina et al., 2003). Our novel glucose uptake results in the presence of docetaxel suggest that the observed lipid accumulation of T47D cells in the presence of MPA+docetaxel is also driven by increased glucose uptake by MPA, underscoring the MPA-activated metabolic pathways for cancer cells survival. Furthermore, glucose is also used to generate, via glycolysis, the glycerol backbone for lipid droplet formation, which is consistent with increased glucose uptake preceding lipid droplet accumulation. Thus, glucose uptake stimulated by MPA fits with the role of progestins as enhancers of the well known glycolytic and lipogenic signature of breast cancer tumors (Szutowicz et al., 1979).

Conclusion

We propose the end results of progestin action are lipid bodies that sequester excess saturated lipid molecules as well as keeping docetaxel away from its site of action on microtubules (Fig. 6). Indeed, docetaxel plus inactivation of SCD-1 resulted in decreased viability of T47D cells as lipid droplets could not be formed to quench the active docetaxel. Our results suggest new combinatorial therapies using chemotherapy agents and natural, edible inhibitors of SCD-1 (like cyclopropene oils) that target lipid-loaded epithelial cancer cells would enhance chemosensitivity in luminal breast cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1 A

ZK98,299

Fig 1 B

Fig 1 C

Figure 1. Progesterone induces lipid accumulation and increases chemoresistance in PR+ breast cancer cells

A, representative photographs of Oil red O stained T47D cells grown on coverslips and treated with or without MPA for 6 days. Docetaxel or ethanol (EtOH) was added for the last

2 days of treatment. Green arrow shows viable cell following docetaxel treatment, black arrows denote apoptotic cells. Lower panels: samples treated as above in the presence of the antiprogesterin, ZK98,299. Lipid staining is absent in the presence of the anti-progesterin. **B**, Quantification of Oil red O staining photographs of T47D and MDA-MB-231 cells using arbitrary density units. White and black bars represent ethanol and MPA treatments respectively. * $p < 0.05$, MPA versus vehicle for each cell line. Bars represent mean $\pm$ SD. Experiments were performed 5 times in independent triplicate assays. **C**, Number of viable T47D and MDA-MB-231 cells after MPA and docetaxel treatments as indicated above. Relative cell viability is shown for each cell line normalized to the untreated control.* $p = 0.02$ vehicle vs. docetaxel for each treatment group (vehicle or MPA). # $p < 0.001$ vehicle versus docetaxel for each treatment group. Experiments were performed 7 times in independent triplicate assays.

Fig. 2A

Fig. 2B

Figure 2. Glucose uptake from the media is increased in progestin-treated cells and required for lipid droplet formation
A, Photographs of Oil red O stains of T47D cells treated with vehicle or MPA with or without glucose. **B**, Glucose uptake of T47D cells treated with ethanol (white bars) or MPA (black bars) for 6 days. The last 2 days of treatment, cells were exposed to docetaxel where indicated. Cytochalasin-B (Cyto-B) was used to block glucose transporters and reveal the transporter-mediated glucose uptake. Bars represent mean $\pm$ SD. Statistically significant values between ethanol and MPA treatment are indicated: * $p < 0.01$, difference between

ethanol (open bars) and MPA. # $p=0.006$, difference between MPA and MPA+insulin treatments. Experiments were performed three independent times in duplicate and one representative experiment is shown.

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Fig. 3A

Fig. 3B

Fig. 3C

Figure 3. Progestin-mediated SCD-1 expression is required for lipid droplet formation and viability of T47D exposed to docetaxel

A, Densitometry of two independent immunoblots for SCD-1 protein normalized to beta-actin is shown. Cells were treated as indicated. Inset; immunoblot for SCD-1 with β -actin as a loading control. **B**, Quantification of Oil red O staining of T47D cells after MPA and docetaxel treatments with or without SCD-1 inhibitor. White and black bars represent ethanol and MPA treatments respectively. * $p < 0.05$, MPA versus vehicle. # $p = 0.006$ compared to MPA+docetaxel. Bars represent mean $\pm$ SEM of 5 independent assays in triplicate. **C**, Number of viable T47D cells after MPA and docetaxel treatments as indicated above. Relative cell viability is shown normalized to the untreated control. \$ $p = 0.02$ compared to vehicle alone. * $p = 0.005$ compared to vehicle alone. # $p = 0.006$ compared to MPA alone. Experiments were performed 7 times in independent triplicate assays.

Fig. 4A

Fig. 4B

Fig. 4C

Figure 4. Progesterin alters the lipid profile of T47D cells
A, Diagram of the site of action of the SCD-1 enzyme. **B**, Gas Chromatography-Mass Spectroscopy (GCMS) analysis of most common fatty acids in the T47D phospholipid fraction: Palmitic (16C:0), palmitoleic (16C:1), stearic (18C:0) and oleic acid (18C:1). Bars represent mean $\pm$ SD. * $p < 0.01$, docetaxel, MPA or MPA+docetaxel treatment versus vehicle. **C**, GCMS of triglyceride fractions (lipid droplets) of T47D lipid extracts. Bars represent mean $\pm$ SD. * $p = 0.04$, docetaxel or MPA+docetaxel treatment versus vehicle. # $p = 0.01$, vehicle versus MPA or MPA+docetaxel treatment.

Figure 5. Liquid chromatography-tandem mass spectroscopy (LCMS/MS) analysis of docetaxel from isolated lipid droplets of T47D cells

Paclitaxel was used as a reference molecule for the analysis. Total lipids from isolated lipid droplets of cells treated with MPA+vehicle (ethanol) or MPA+docetaxel (100nM) were extracted using solid-phase cartridges and analyzed by LC/MS/MS. The chromatograms show the ion intensity profiles for docetaxel (solid line) and paclitaxel (dashed line).

Figure 6. Diagram of the proposed mechanism of progestin action in the lipid profile and survival of T47D cells exposed to docetaxel

SCD-1 increases membrane fluidity and induces the formation of lipid droplets that are able to sequester active docetaxel molecules, favoring cell survival. SCD-1 inhibition leads to lack of lipid droplets, increased free docetaxel and decreased cell viability as well as increases membrane fluidity in the FASN, fatty acid synthase. SCD-1, steroyl CoA desaturase. PL, phospholipid. LD, lipid droplets.

Table of saturation content of the triglycerides (lipid droplets) and the phospholipids fractions of T47D cells treated with MPA for 6 days with or without docetaxel for the last 48 hours.

Table 1

Table of saturation content %($\pm$ SD)		
	PL	LD
vehicle	50.59 ($\pm$ 1.64)	74.88 ($\pm$ 0.16)
MPA	33.14 ($\pm$ 0.31) [#]	80.59 ($\pm$ 4.44)
docetaxel	38.79 ($\pm$ 0.05) [*]	82.23 ($\pm$ 1.69)
MPA+docetaxel	33.23 ($\pm$ 3.73) [*]	80.06 ($\pm$ 2.11)

^{*} Significantly different from vehicle (p<0.01)

[#] Significantly different from docetaxel (p<0.02)

LD= Lipid Droplet; PL= Phospholipids