Molecular Modulation of Estrogen-Induced Apoptosis by Synthetic Progestins in Hormone Replacement Therapy: An Insight into the Women's Health Initiative Study

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Abstract

Hormone replacement therapy (HRT) is widely used to manage menopausal symptoms in women and can be comprised of an estrogen alone or an estrogen combined with a progestin. The Women’s Health Initiative demonstrated in their randomized trials that estrogen alone HRT decreases the risk of breast cancer in postmenopausal women, whereas combined estrogen plus a progestin (medroxyprogesterone acetate, MPA) HRT increases this risk. Long-term estrogen-deprived MCF-7:5C cells were used to model the postmenopausal breast cancer cell environment. MPA is able to modify E₂-induced apoptosis in MCF-7:5C cells. MPA, similar to dexamethasone, increases glucocorticoid receptor (GR) transcriptional activity, increases SGK1, a GR target gene, and can be blocked by RU486 (an antiglucocorticoid), suggesting that it functions through the GR. Norethindrone acetate (NETA), another progestin used in HRT, acts like an estrogen at high doses, upregulating estrogen receptor target genes and generating apoptosis in MCF-7:5C cells. The data suggest that women taking HRT comprising an estrogen plus MPA may have an increased risk of breast cancer due to MPA acting as a glucocorticoid and blunting E₂-induced apoptosis in this environment. Therefore, perhaps other approved progestins (e.g., NETA) should be considered as alternatives to MPA. Cancer Res; 74(23); 1–9. ©2014 AACR.

Introduction

Conjugated equine estrogen (CEE) was introduced into clinical practice in 1941 for the treatment of menopausal symptoms and related conditions. Despite its widespread use, there were concerns with unopposed estrogen therapy. Reports (1, 2) of an increased risk of endometrial cancer in women taking CEE resulted in package label warnings about the risks of cancer and thrombosis. A solution was required for the long-term use of CEE to treat osteoporosis in postmenopausal women without risking endometrial cancer. Histologic examination of biopsies from women taking CEE showed proliferation of the endometrial lining, but the addition of a synthetic progestin prevented this action (3). The FDA approved the use of a combination of an estrogen and a synthetic progestin for the treatment and prevention of osteoporosis in 1986. The combination of CEE and a synthetic progestin, here medroxyprogesterone acetate (MPA), is referred to as hormone replacement therapy (HRT).

Observational clinical and laboratory studies supported the view that HRT users had a reduced risk of coronary heart disease and atherosclerosis (4, 5). These encouraging data led to randomized clinical trials, the largest of which is the Women's Health Initiative (WHI) comprising two parallel studies that enrolled a total of 27,000 subjects. Women with an intact uterus were randomized to placebo or HRT (CEE 0.625 mg and MPA 2.5 mg daily), and hysterectomized women were randomized to placebo or CEE alone. The trial was stopped when the HRT arm exceeded the predefined safety limit for the risk of breast cancer, as well as heart disease, stroke, and clot clots (6). Unexpectedly, however, the CEE alone group reported decreased breast cancer incidence and mortality after stopping this trial 18 months later for an increase in strokes (7).

Laboratory studies over the past decade have documented and deciphered a new biology of estrogen-induced apoptosis that occurs in long-term estrogen-deprived breast cancer cells (8, 9). These studies translate to benefit in clinical trials of estrogen therapy in antihormone-resistant breast cancer (10, 11). The question becomes: if the molecular mechanism of estrogen-induced apoptosis is defined in well-documented laboratory models of estrogen-deprived breast cancer cells (12–14), why does a combination of MPA plus CEE in the WHI increase the risk of breast cancer (15)? Although there is a large body of experimental evidence that the female sex hormones estrogen and progesterone are responsible for breast cancer growth (16), the paradox that estrogen induces apoptosis in estrogen-deprived breast cancer cells (10) has created a new dimension in our understanding of physiologic estrogen action in a woman’s body.
The central question to be addressed is whether a synthetic progestin, MPA, can modulate estrogen-induced apoptosis and cause breast cancer cell growth. Here, we demonstrate that different synthetic progestins have different pharmacologic actions that exert different selection pressures during long-term therapy in culture. We report for the first time that the glucocorticoid properties of MPA (17) are responsible for blunting the apoptotic actions of estrogen, resulting in the growth of a new breast cancer cell population that is better able to survive.

Materials and Methods

Cell culture

MCF-7:5C and MCF-7:WS8 cell lines were cultured in phenol red-free RPMI-1640 media supplemented with 10% charcoal-stripped fetal bovine serum. Media and treatments were replaced every 3 days. DNA fingerprinting patterns of the cell lines are consistent with the report by the ATCC (18). The MCF-7:5C cell line was chosen for its representation of the estrogen-deprived breast cancer cell and its ability to undergo estrogen-induced apoptosis; the MCF-7:WS8 cell line represents the estrogen-fueled breast cancer cell environment. Estradiol (E2; Sigma-Aldrich), dexamethasone (Dex; Sigma-Aldrich), MPA (Sigma-Aldrich), norethindrone acetate (NETA; Sigma-Aldrich), R5020 (Sigma-Aldrich), RU486 (Sigma-Aldrich), 4-hydroxytamoxifen (4-OHT; Sigma-Aldrich), ICI 182,780 (ICI; Sigma-Aldrich), and combinations were dissolved in ethanol and then in media. MPA and NETA were chosen as two representative progestins used in HRT.

Cell proliferation assay

MCF-7:5C cells were harvested after treatment with vehicle (0.1% ethanol), E2 (10⁻⁹ mol/L, 1 nmol/L), Dex (10⁻⁶ mol/L, 1 μmol/L), MPA (10⁻⁶ mol/L, 1 μmol/L), NETA (10⁻⁶ mol/L, 1 μmol/L), R5020 (10⁻⁶ mol/L, 1 μmol/L), RU486 (10⁻⁶ mol/L, 1 μmol/L), 4-OHT (10⁻⁶ mol/L, 1 μmol/L), or combinations, in triplicate, for specified time. Media and treatments were replaced every 3 days. DNA content was measured as using the Fluorescent DNA Quantitation Kit (Bio-Rad).

Immunoblotting

ERα (sc-544), GR (glucocorticoid receptor; sc-8892), and PR (progesterone receptor; sc-810) antibodies were purchased from Santa Cruz Biotechnology, PARP (#9532S) antibody from Cell Signaling Technology, and β-actin antibody (A5441) from Sigma-Aldrich. Proteins were harvested from cells in 10-cm dishes using cell lysis buffer (Cell Signaling Technology) supplemented with Protease Inhibitor Cocktail Set I and Phosphastrate Inhibitor Cocktail Set II (Calbiochem). Bicinchoninic acid assay was used to quantify total protein content (Bio-Rad Laboratories). Protein (50 μg) was probed and visualized as previously described (18).

Annexin V analysis of apoptosis

Apoptosis was quantified by flow cytometry using the FITC-labeled Annexin V (FL1-H) and PI (FL2-H). Cells were analyzed using FACSsort flow cytometer (Becton Dickinson).

Quantitative real-time RT-PCR

Cells were harvested from 6-well plates using TRIzol, and RNA was isolated using the RNeasy Micro Kit (Qiagen). RNA was reverse transcribed using an Applied Biosystems kit. SYBR green (Applied Biosystems) was used for quantitative real-time PCR (RT-PCR) in triplicate in a 7900HT Fast Real-Time PCR system (Applied Biosystems).

GRE activity

Transient transfection assay was conducted using a dual-luciferase system (Promega). To determine GR transcriptional activity, cells were transfected in 24-well plates for 24 hours with a glucocorticoid response element (GRE)–regulated dual-luciferase reporter plasmid (gift from Dr. Anne Gompel, Université Paris Descartes, Paris, France) or an estrogen response element (ERE)–regulated dual-luciferase reporter plasmid (gift from Dr. Rebecca Riggins, Georgetown University, Washington, DC). Cells were treated with specified compounds for 24 hours following transfection, in triplicate. Cells were then harvested and processed for dual-luciferase reporter activity, in which firefly luciferase activity was normalized by Renilla luciferase activity.

Statistical analysis

Values reported are means ± SEM. Significant differences were found by the Student t test. P values <0.05 were considered statistically significant.

Results

MCF-7:5C growth patterns with HRT compounds

MCF-7:5C cells represent a stable cell line derived from parental MCF-7 cells by long-term estrogen deprivation, and are the relevant cells used in the following studies. They are estrogen receptor (ER)–positive, GR-positive, and PR-negative (Supplementary Fig. S1A). MCF-7:5C cells were treated for 8 days with a vehicle, R5020, Dex, MPA, or NETA (another progestin used in HRT; Fig. 1A). R5020 was used as a pure progestin-positive control and had no effect on growth. After 8 days, Dex and MPA caused a 28.7% and 21.6% decrease in MCF-7:5C cell DNA, respectively, compared with vehicle treatment. NETA, however, caused a 93.6% decrease in MCF-7:5C cells. Dose–response assays were carried out with various concentrations of the compounds in both MCF-7:5C (Supplementary Fig. S2) and in the parental estrogen-sensitive MCF-7:WS8 cells (Supplementary Figs. S3 and S7).

MCF-7:5C cells were then treated longer with the compounds (Fig. 1B). Again, cells treated with Dex and MPA do not grow as quickly as the control cells. NETA and estradiol (E2) both cause MCF-7:5C cell death; minimal DNA is sustained for 6 weeks of treatment. To mimic HRT, MCF-7:5C cells were treated with combinations of E2+MPA and E2+NETA, and compared with vehicle, E2 alone, and E2+Dex (Fig. 1C). Cells treated with 1 nmol/L E2 die during the first week of treatment, but Dex and MPA seem to reverse this effect; cells are able to...
grow. E₂ + NETA trigger the same death response as E₂ alone, suggesting NETA’s role as an estrogen. Also, NETA decreases MCF-7:5C ER levels similarly to the effect of E₂ after 2 months treatment (Supplementary Fig. S1B). NETA caused the growth of MCF-7:WS8 cell and this was blocked by the 4-OHT or fulvestrant (Supplementary Fig. S3C).

**Dex and MPA block E₂-induced apoptosis in MCF-7:5C**

MCF-7:5C cells underwent these same treatments for 72 hours, and Annexin V staining was measured by flow cytometry to indicate apoptosis (Fig. 2A). E₂ caused 29.03 (±1.44)% of cells to undergo apoptosis. Dex was able to block this apoptosis as shown by E₂ + Dex causing only 5.37 (±0.35)% of cells to stain for Annexin V. NETA and E₂ + NETA show similar Annexin V staining as E₂ alone; both treatments cause apoptosis (30.17 ± 0.65 and 33.23 ± 0.97%, respectively). Although MCF-7:5C cells are eventually able to grow under E₂ + MPA treatment (Fig. 1C), MPA is not able to block initial E₂-induced apoptosis at 72 hours; more time is required for MPA to exert its subtle long-term effect. When Poly (ADP-ribose) polymerase (PARP) cleavage is probed by Western blot analysis to indicate apoptosis, again we show that E₂ alone, and in combination with MPA, causes apoptosis after 72 hour treatments. However, MPA can block E₂-induced apoptosis after 6, 9, and 12 days of combination treatment, as shown by decreased cleaved PARP protein expression. This confirms the biologic effect already seen, as MCF-7:5C cells can grow in the presence of E₂ + MPA (Fig. 2B).

After a 2-month treatment, microscopy photographs were taken to illustrate the dramatic effects Dex and MPA have on E₂-treated cells. Cells treated with both E₂ and E₂ + NETA show a reduction in MCF-7:5C cell number. E₂ + Dex and E₂ + MPA treatments both allow MCF-7:5C cells to grow during the 2 months of treatment as demonstrated by increased cell number seen in the photographs (Fig. 2C).

**NETA functions as an estrogen at high concentration**

To illustrate estrogenic actions of NETA, MCF-7:5C cell DNA was measured after treatment with the drugs alone and in combination with 4-OHT, an antiestrogen. As a positive control, the data confirm that 4-OHT is able to reverse E₂’s apoptotic action as shown by significantly increased DNA when MCF-7:5C cells are treated with combination compared with E₂ alone. 4-OHT also has the ability to reverse NETA’s decrease in MCF-7:5C DNA, suggesting its role as an estrogen (Fig. 3A). Estrogen target gene mRNA expression was then measured in MCF-7:WS8 cells by RT-PCR. Whereas Dex and MPA generate no increase in estrogen target genes pS2 (Fig. 3B) or PR (Supplementary Fig. S4), NETA elicits significant upregulation of both pS2 (23.6- and 46.9-fold) and PR (30.6- and 81.0-fold) at the two higher concentrations (Fig. 3B and Supplementary Fig. S4). This result is similar to that of E₂, which increases pS2 64.9-fold and PR 58.9-fold over vehicle (Fig. 3B and Supplementary Fig. S4) in MCF-7:WS8 cells after 24 hour treatment. Furthermore, when MCF-

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*Figure 1. MCF-7:5C growth with HRT compounds over time. A, MCF-7:5C cells were treated with vehicle or 1 μmol/L concentrations of R5020, Dex, MPA, or NETA. Cells were harvested every 2 days for 8 days, and DNA was quantified. B, MCF-7:5C cells were treated with vehicle, 1 nmol/L E₂, or combinations of 1 nmol/L E₂ plus 1 μmol/L Dex, MPA, or NETA. Cells were harvested and DNA was quantified every 3 days until the cells reached confluence, up until 42 days. C, MCF-7:5C cells were treated with vehicle, 1 nmol/L E₂, or combinations of 1 nmol/L E₂ plus 1 μmol/L Dex, MPA, or NETA. Cells were harvested and DNA was quantified every 3 days until the cells reached confluence, up until 42 days. Means represent samples in triplicate. ***, P < 0.001, compared with vehicle; **, P < 0.01, compared with E₂ + NETA.*
7:5C cells are transiently transfected with an ERE–luciferase construct, NETA is able to generate 8.12-fold higher ERE activity than vehicle-treated cells (Fig. 3C). This increased ERE activity is blocked by ICI (Fig. 3C) and 4-OHT (Supplementary Fig. S5), suggesting its reliance on the ER. Similar ERE activity results were generated in MCF-7:5C cells (Supplementary Fig. S6).

MPA functions as a glucocorticoid through GR similarly to Dex

To classify MPA as a glucocorticoid similar to Dex, MCF-7:5C cell DNA was quantified after treatment with the drugs alone and in combination with RU486, an anti-glucocorticoid. Dex and MPA alone cause a reduction in MCF-7:5C cell DNA; however, RU486 can reverse this reduction when treated in combination (Fig. 4A). These data suggest that MPA could be working through the GR in these cells.

To further test glucocorticoid behavior of MPA, GR target gene SGK1 mRNA was quantified by RT-PCR. Dex and MPA dramatically increased SGK1 expression in a time-dependent manner (Fig. 4B). This upregulation was inhibited by at least 50% when RU486 was added in combination to treatment, suggesting a GR-mediated mechanism. Furthermore, GRE activity was detected by transiently transfecting MCF-7:5C cells with a GRE–luciferase reporter vector. The cells were then treated for 24 hours with a vehicle, RU486, R5020, Dex, MPA, NETA, or combinations. Cells treated with Dex induced 289.39 (±0.11)-fold GRE activity over vehicle-treated cells, confirming the validity of the assay. Importantly, MPA also caused 99.44 (±0.01)-fold more GRE activity compared with vehicle, indicating its ability to activate GR transcription (Fig. 4C). GRE activity generated by both Dex and MPA was blocked by RU486 when treated in combination (Fig. 4C). NETA was unable to elicit a dramatic increase in GRE activity (Fig. 4C).
Dex and MPA block E2-induced apoptosis-related genes similarly

To explore pathways involved in Dex and MPA blocking E2-induced apoptosis, RT-PCR was performed using primers for genes associated with E2-induced apoptosis. E2 triggers apoptosis in MCF-7:5C cells by increasing levels of TNFα, HMOX1, LTA, and LTB after 72 hours of treatment. Both Dex and MPA are able to significantly decrease upregulation of these genes in response to E2 when treated in combination (Fig. 5). This suggests that MPA can work in a similar way as Dex by blocking key genes necessary for E2 to initiate apoptosis in this setting.

Discussion

The hypothesis addressed in this study is whether a synthetic progestin in HRT that exhibits glucocorticoid activity can interfere with E2-induced apoptosis in breast cancer cells. The results of the WHI indicate an increase in the risk of breast cancer in women taking CEE + MPA (15); MPA was, therefore, expected to increase the growth of breast cancer cells in the presence of E2 in our well-studied cellular model, and was predicted to function as a glucocorticoid. To test this hypothesis, Dex was used as a known glucocorticoid-positive control that can block E2-induced apoptosis (Fig. 2A). This classification of Dex is consistent with reports in the literature that indicate that glucocorticoids can prevent apoptosis in various contexts of cancer (19–21).

Reports in the literature demonstrate the potential of synthetic progestins to bind and activate other nuclear receptors than their own cognate receptors. NETA and other 19-nortestosterone derivatives have been shown to activate the ER and stimulate the growth of estrogen-responsive MCF-7 and T47D.
The estrogenic activity of NETA is confirmed in this work through its ability to increase cell replication (DNA) in MCF-7:WS8 cells (Supplementary Fig. S3C), to elevate estrogen target genes (Fig. 3B, Supplementary Fig. S4), generate apoptosis in MCF-7:5C cells (Fig. 2A), increase ER transcriptional activity (Fig. 3C, Supplementary Figs. S5 and S6), and has its action be blocked by 4-OHT and ICI (Fig. 3A and C).

Notably, reports have indicated that the synthetic progestin, MPA can bind to and activate not only the PR, but also the GR in breast cancer (24). A more recent report shows that MPA has affinity for the GR and can compete with the natural glucocorticoid, cortisol, in the body (25). Others have recently demonstrated that MPA can increase glucocorticoid activity in MCF-7 cells (26).

The data presented in this study integrate the previously published findings and establish that MPA functions as a glucocorticoid in long-term estrogen-deprived breast cancer cells, blocking E2-induced apoptosis and allowing cells to grow (Fig. 1C). MPA cannot block E2-induced apoptosis as early as Dex (Fig. 2A); it requires at least 6 days to gain sufficient glucocorticoid activity (Fig. 2B and C). When MPA is able to inhibit E2-induced apoptosis, like Dex, it acts by preventing upregulation of important E2-induced apoptosis-related genes (Fig. 5). We have noted previously that Dex decreases the growth of MCF-7:5C cells and blocks E2-induced apoptosis (27). Dex blocks E2-stimulated growth of MCF-7:WS8 cells (Supplementary Fig. S7). MPA prevents E2-stimulated growth at high concentration (10^{-6} mol/L), with similar effect as the more potent Dex in wild-type MCF-7:WS8 cells (Supplementary Fig. S7). This inhibition is not an effect of the progestin action of MPA as R5020, a progestin with no glucocorticoid action has no effect on E2-stimulated MCF-7:WS8 growth (Supplementary Fig. S7A).
Although others have demonstrated MPA’s function as a glucocorticoid (24–26), it is illustrated here as a mechanistic explanation for the increased breast cancer risk observed in the CEE+MPA arm of the WHI clinical trials (15). By modeling the environment of postmenopausal breast cancer using long-term estrogen-deprived MCF-7:5C cells, we can predict clinical responses through laboratory experiments. In so doing, we propose a unifying hypothesis of the modulation of E2-induced apoptosis to explain the results of the WHI HRT trial in populations of postmenopausal women over the age of 60 years (15).

Critical to the understanding of how these effects occur, studies describe the interplay between ER and GR, and how AP-1 can integrate their transcriptional responses (28). Recent reports (29) also show that the ER and GR can interact and cause differential activation by reconfiguring the chromatin structure at GRE or ERE sites in the DNA. Furthermore, it is shown that GR can inhibit ER transcriptional activity and ER-mediated proliferation in breast cancer (30). Consistently, Dex blocks E2-stimulated MCF-7:WS8 cell replication (Supplementary Fig. S7). We can speculate that when MPA binds to the GR, the complex binds to GREs in the DNA, which then affects the ability of ligand-bound ER to functionally transcribe ER target genes. In order for E2-induced apoptosis to occur, E2 binds to ER, resulting in transcription of genes (e.g., HMOX1, TNFα, etc.) necessary for apoptosis. When this process is inhibited, possibly by the chromatin remodeling actions of MPA-bound GR, apoptosis is decreased even in an estrogenic setting.

Inflammation is also critical for MCF-7:5C cells to undergo apoptosis; it has been shown that E2 upregulates key inflammatory genes in these cells (11). Glucocorticoids function to block inflammation, and are used in the clinic as anti-inflammatory drugs. Dex and MPA may prevent E2-induced apoptosis by downregulating proinflammatory genes (e.g., IFNL1, BCL10, IL4R, FADS1, etc.) through binding to GR.
It is important to consider timing when considering the implications of this work; timing of HRT can make a dramatic difference in response to treatment (31, 32). MCF-7/SC cells represent long-term estrogen-deprived cells, we sought this is the biologic context required in the patient as well to replicate the patient population in the WHI CEE alone trial (15). Previous studies have examined how a ‘gap’ is needed after menopause to sufficiently deprive women’s nascent breast cancer cells of estrogen (33). Five to 10 years postmenopause is appropriate to ensure effective apoptosis when CEE is introduced to the woman (31). Without this delay, exogenous estrogen may stimulate breast cancer growth, resulting in increased breast cancer risk; in fact, studies show that breast cancer risk is greater when HRT is initiated closer to menopause (34).

The findings presented can have clinical impact as CEE+MPA is frequently taken by postmenopausal women to alleviate menopausal symptoms. Patients should perhaps be advised to choose a different approach with a selective ER modulator (SERM)/CEE combination (35), and also to delay HRT until they are appropriately past menopause. Because the synthetic progestin NETA can prevent endometrial cancer, and also cause breast cancer apoptosis like an estrogen, it appears to be a logical alternative to MPA as an alternative approved synthetic progestin that could be used in HRT.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: P. Fan, V.C. Jordan Development of methodology: E. Sweeney, P. Fan Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Sweeney, P. Fan, V.C. Jordan Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Sweeney, P. Fan Writing, review, and/or revision of the manuscript: E. Sweeney, V.C. Jordan Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Fan Study supervision: P. Fan, V.C. Jordan

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