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Opposing actions of the progesterone metabolites, 5α-dihydroprogesterone (5αP) and 3α-dihydroprogesterone (3αHP) on mitosis, apoptosis, and expression of Bcl-2, Bax and p21 in human breast cell lines

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ABSTRACT

Previous studies have shown that breast tissues and breast cell lines convert progesterone (P) to 5α-dihydroprogesterone (5αP) and 3α-dihydroprogesterone (3αHP) and that 3αHP suppresses, whereas 5αP promotes, cell proliferation and detachment. The objectives of the current studies were to determine if the 5αP- and 3αHP-induced changes in cell numbers are due to altered rates of mitosis and/or apoptosis, and if 3αHP and 5αP act on tumorigenic and non-tumorigenic cells, regardless of estrogen (E) and P receptor status. The studies were conducted on tumorigenic (MCF-7, MDA-MB-231, T47D) and non-tumorigenic (MCF-10A) human breast cell lines, employing several methods to assess the effects of the hormones on cell proliferation, mitosis, apoptosis and expression of Bcl-2, Bax and p21. In all four cell lines, 5αP increased, whereas 3αHP decreased cell numbers, [^{3}H]thymidine uptake and mitotic index. Apoptosis was stimulated by 3αHP and suppressed by 5αP. 5αP resulted in increases in Bcl-2/Bax ratio, indicating decreased apoptosis; 3αHP resulted in decreases in Bcl-2/Bax ratio, indicating increased apoptosis. The effects of either 3αHP or 5αP on cell numbers, [^{3}H]thymidine uptake, mitosis, apoptosis, and Bcl-2/Bax ratio, were abrogated when cells were treated simultaneously with both hormones. The expression of p21 was increased by 3αHP, and was unaffected by 5αP. The results provide the first evidence that 5αP stimulates mitosis and suppresses apoptosis, whereas 3αHP inhibits mitosis and stimulates apoptosis. The opposing effects of 5αP and 3αHP were observed in all four breast cell lines examined and the data suggest that all breast cancers (estrogen-responsive and unresponsive) might be suppressed by blocking 5αP formation and/or increasing 3αHP. The findings further support the hypothesis that progesterone metabolites are key regulatory hormones and that changes in their relative concentrations in the breast microenvironment determine whether breast tissues remain normal or become cancerous.

1. Introduction

Previous studies have shown that breast tissues convert progesterone to 5α-pregnane-3,20-dione (5α-dihydroprogesterone; 5αP) and 3α-hydroxy-4-pregnen-20-one (3α-dihydroprogesterone; 3αHP) and that tumorous tissues produce higher levels of 5αP and lower levels of 3αHP than normal breast tissues [1]. These differences in progesterone metabolism between normal and tumorous tissues were observed in all breast tissues examined, regardless of the age of the women, subtypes and grades of carcinomas, and whether the tissues were estrogen receptor (ER) and progesterone receptor (PR) positive and/or negative [1]. In addition, measurements of actual amounts of 5αP and 3αHP using radioimmunoassays and mass spectrometry also showed that the ratio of 5αP:3αHP is markedly higher in tissues and nipple aspirate fluid from tumorous than from nontumorous breast [1,2]. Similarly tumorigenic (MCF-7, MDA-MB-231, T47D) breast cell lines convert more progesterone to 5αP and less to 3αHP than non-tumorigenic (MCF-10A) cells [3]. The enzymes responsible for conversion of progesterone to 5αP and 3αHP are 5α-reductase and 3α-hydroxysteroid oxidoreductase (3α-HSO), respectively, and the higher ratio of 5αP:3αHP in neoplastic breast tissues and tumorigenic breast cell lines result from higher 5α-reductase and/or lower 3α-HSO gene expression and activities [3,4]. The general scheme of progesterone metabolism to 3αHP and 5αP is presented in Fig. 1.

The progesterone metabolism studies on human breast tissues and cell lines suggest that increases in 5αP and decreases in 3αHP production accompany the shift toward breast cell tumorigenicity and neoplasia. Indeed, *in vitro* studies [1] on human breast cell

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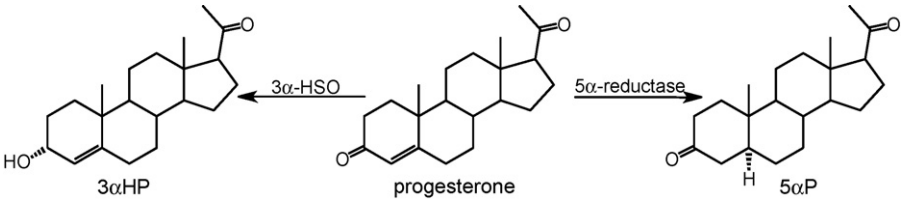

Fig. 1. General scheme of progesterone conversion to 3αHP and 5αP in breast tissues and human breast cell lines. (3α-HSO, 3α-hydroxysteroid oxidoreductase).

lines have shown that proliferation is significantly increased by 5αP and decreased by 3αHP. The opposing effects on proliferation are dose-dependent (10^{-8} to 10^{-6} M), occurring at concentrations found in breast tissues and nipple aspirate fluid ($1–5 \times 10^{-6}$ M) [2], and have been demonstrated in tumorigenic and non-tumorigenic, as well as ER/PR positive and negative human breast cell lines [1,2].

The opposing effects of 5αP and 3αHP have also been observed with respect to cell adhesion and regulation of cytoskeletal and adhesion molecules: 5αP stimulates cell detachment, increases actin depolymerization and decreases vinculin-containing adhesion plaques, whereas 3αHP inhibits cell detachment and increases actin polymerization and adhesion plaques [5]. The opposing effects of 5αP and 3αHP are mediated via specific receptors located on the plasma membranes of human breast cells [6] and the ligand–receptor interactions trigger cell signaling processes [7]. Radioisotopic studies indicate separate and distinct receptors, some with high affinity and specificity for 5αP (5αPR) and others with high affinity and specificity for 3αHP (3αHPR) [6,8]. The receptors exhibit only negligible affinity for other steroids such as estradiol-17β, progesterone and its other metabolites, androgens and corticosteroids [6]. In addition to various opposing actions with respect to adhesion and proliferation, 5αP and 3αHP also have opposing effects on the regulation of 5αPR [8] and ER [9] numbers.

Increases or decreases in the net rate of cell proliferation are determined by the relative rates of cell replication (mitosis) and programmed cell death (apoptosis), or both. The objectives of the current studies were to determine (a) if the effects on proliferation are due to actions on apoptosis and/or mitosis, (b) if the effects of 5αP are abrogated by 3αHP and vice versa, and (c) if the effects can be demonstrated on breast cell lines with different characteristics. The studies were conducted on four different cell lines. Three of the cell lines (MCF-7, MDA-MB-231, T47D) are known to be tumorigenic in immunosuppressed mice [10,11]; among these, MCF-7 and T47D cells are ER and PR positive [12] and estrogen or progesterone dependent for tumorigenicity, whereas MDA-MB-231 cells are ER and PR negative and develop tumors spontaneously without steroid hormone supplements. The fourth cell line, MCF-10A, is ER and PR negative and considered to be non-tumorigenic [13]. Apoptosis was assessed by evaluating nuclear morphology, DNA fragmentation (TUNEL) assay and annexin V binding.

As well, the effects of 5αP and 3αHP on expression of bcl-2 and bax genes, whose encoded protein products are known to either promote or inhibit release of apoptosis-inducing cytochrome *c* from the inter-membrane space of mitochondria [14,15], and on the expression of p21, a cell cycle inhibitor [16,17], were examined in MCF-7 cells.

The results are the first to show that 5αP increases cell proliferation primarily by stimulating mitosis but also by countering pro-apoptotic stimuli, while 3αHP primarily increases apoptosis but also suppresses mitosis in stimulated cells, and the actions involve changes in expression of bcl-2, bax and p21 genes. The effects of 5αP are abrogated by 3αHP, and vice versa. The effects of 5αP and 3αHP on mitosis and apoptosis were observed in all four cell lines regardless of tumorigenicity and ER/PR status.

2. Materials and methods

2.1. Chemicals

3αHP was obtained from Steraloids (Newport, RI). 5αP, cell culture media, insulin, penicillin and streptomycin were obtained from Sigma Chemical Co. (Oakville, ON). Serum was purchased from Invitrogen (Burlington, ON). [^{3}H]thymidine (methyl-^{3}H-thymidine; 48 Ci/mmol) was obtained from Amersham Biosciences (Piscataway, NJ). Other chemicals and solvents were of appropriate analytical grade and were purchased from Sigma Chemical Co., BDH Inc., (Toronto, ON), VWR (Mississauga, ON) or Fisher Scientific Ltd. (Toronto, ON).

2.2. Cell culture

Tumorigenic (MCF-7, MDA-MB-231, T-47D) and non-tumorigenic (MCF-10A) human breast cell lines were obtained from American Type Culture Collection (Manassas, VA). The cells were grown in a 1:1 Ham's F12 Medium and Dulbecco's Modified Eagle's Medium supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 78 units/ml streptomycin, 10 μg/ml insulin, 1.2 mg/ml sodium bicarbonate and 10% calf serum as previously described [1,3]. Cells were grown in T-75 flasks (Sarstedt) in a humidified incubator at 37 °C and a 5% CO_2 atmosphere and were harvested at approximately 80% confluence. The harvested cell were then seeded and treated as described below. Cell viability was determined by the trypan blue exclusion test.

2.3. Cell treatments

The progesterone metabolites, 5αP and 3αHP, were dissolved at 10^{-3} M in double glass-distilled ethanol and added to media containing 5% charcoal stripped fetal bovine serum at a final concentration of 10^{-6} M and ethanol at 0.1%. Treatment media were prepared at least 24 h prior to use to assure complete and uniform dissolution of steroids. Control medium contained 0.1% ethanol without steroids.

2.4. Cell proliferation

Cell treatments were as described [1,5]. Cells were seeded in 24-well plates (Nunclon) at about 4×10^4 cells/well (for hemocytometer counts) and in 60 mm Petri dishes (Sarstedt) at 5×10^4 cells/dish (for [^{3}H]thymidine uptake) and were allowed to attach for 24 h. Medium was removed and cells were cultured in medium containing 5% charcoal stripped serum without (control) or with 10^{-6} M 5αP, 3αHP or 5αP + 3αHP. Treatments were for 72 h and treatment media were replaced every 24 h. Each treatment had 4–6 replicate dishes and experiments were repeated at least 3 times. At termination, cell proliferation was determined by cell counts using a hemocytometer or by [^{3}H]thymidine uptake. For [^{3}H]thymidine uptake determinations, the following procedure was applied. On the day of termination, 0.5 μCi [^{3}H]thymidine was added to each dish, and the incubation continued for 6 h. The

incubations were terminated with two washes with cold (4 °C) PBS and the addition of 1 ml of 10% trichloroacetic acid. After 20 min at 4 °C, dishes were washed 2 times with 1 ml absolute ethanol, and then incubated with 1 ml of 1.0N NaOH at 37 °C for 30 min to solubilize the cell lysates. An aliquot (0.5 ml) of each lysate was used to determine the incorporated radioactivity by scintillation spectrometry (Beckman LS 6500).

2.5. Mitosis

For each cell line, about 10^4 cells were seeded on 22 mm × 22 mm acid-treated coverglasses (5–8 per treatment) and allowed to attach for 24 h. They were then treated without (control), or with 10^{-6} M 5αP, 3αHP, or 5αP + 3αHP. At termination, cells were fixed in 2% formaldehyde and stained with Hoechst 33258 (Sigma). The coverglasses were mounted on slides using fluorescent mounting medium, and the preparations were analyzed by fluorescence microscopy (usually within 24 h). Sixteen fields were chosen from each coverglass, according to a designated objective pattern, four fields in each row and four fields in each column, from top row to bottom row, and from left to right. For each field, the total number of nuclei, and the number undergoing mitosis, was determined and the percent of cells with mitotic nuclei per coverglass was calculated. For each treatment, 90–100 fields and about 1500–2000 cells in total were examined.

2.6. Apoptosis

Cells (about 10^4) were seeded on 22 mm × 22 mm acid-treated coverslips (5–8 per treatment) and treated as described under Section 2.5.

2.6.1. Annexin V staining

Annexin V binds to phosphatidylserine that is translocated to the outer leaflet of the plasma membrane of cells in the early stages of apoptosis. Annexin V staining was conducted with the use of an Annexin V-Cy3 Apoptosis Detection Kit (Sigma) according to the manufacturer's protocol. The coverglasses were then mounted on glass slides and immediately viewed under a fluorescent microscope. For each treatment at least 5 coverslips (a total of 90–100 fields) and about 1500–2000 cells in total were examined.

2.6.2. Apoptosis analysis using Hoechst staining

Cells were fixed and stained as for the mitosis studies, and all cells in each field were counted; those nuclei showing definite chromatin condensation were scored as apoptosis positive. The percent of cells with apoptotic nuclei per coverslip was determined. For each treatment at least 5 coverslips (a total of 90–100 fields) and about 1500–2000 cells in total were examined.

2.6.3. TUNEL assays

TUNEL assays (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling) were performed using an *In Situ* Detection Kit (Boeringer), according to the manufacturer's protocol. Following termination procedures, the coverglasses were sealed onto the slides with water-soluble mounting solution, appropriate fields were selected as described under Section 2.5, and the percent cells undergoing apoptosis was determined using light microscopy.

2.7. Analysis of bcl-2, bax and p21 by real-time PCR

MCF-7 cells were seeded in 6-well plates at about 50,000 cells/well and were allowed to attach for 24 h. Medium was removed and cells were cultured in medium containing 5% charcoal stripped serum without (control) or with 10^{-6} M 5αP, 3αHP or 5αP + 3αHP. Treatments were for 72 h and treatment media were replaced every 24 h. Each treatment had four replicate dishes and experiments were repeated 4 times.

Total RNA was extracted from cells using TRIzol® reagent (Invitrogen) following the manufacturer's protocol. Total RNA was quantified by spectrophotometry, assuming absorbance at 260 nm of 1.0 equal to 40 μg RNA/ml. Reverse transcription with Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Invitrogen) was preformed to obtain complementary DNA (cDNA).

Real-time PCR was conducted with a Rotor-Gene temperature cycler (Corbett Research) using cDNA as a template and Platinum® SYBR® Green qPCR SuperMix UDG (Invitrogen), following the manufacturer's protocol. PCR primer sets for BAX (Bax), BCL2 (Bcl-2), and CDKN1A (p21) (SuperArray Bioscience Co., Frederick, MD) were used to measure expression levels. For each sample, measures of gene expression were normalized as a function of the expression of the 18S ribosomal RNA; 18S primer sequences (Invitrogen) were the same as those previously published [3]. Reverse transcription controls (no MMLV) and non-template controls (no cDNA) were included in the PCR cycling to ensure that genomic DNA was not amplified and reagents were not contaminated.

2.8. Statistical analyses

For the cell proliferation (cell number, [^{3}H]thymidine uptake), mitosis and apoptosis studies, results are expressed in relation to the controls. For the Bcl-2, Bax and p21 expression studies, the results were standardized in relation to 18S rRNA expression and the overall results from the four replicate experiments were transformed to Bcl-2/Bax ratios and are presented with respect to the controls (100%). Results are presented as mean ± SEM and were analyzed by one-way ANOVA followed by Tukey–Kramer comparisons test to detect differences between groups ($p < 0.05$), using the computer program InStat (GraphPad, San Diego, CA).

3. Results

3.1. Effect of 5αP and 3αHP on proliferation of human breast cell lines

Dose–response effects of 5αP and 3αHP were demonstrated for each cell line with respect to cell counts and [^{3}H]thymidine incorporation. 5αP resulted in dose-dependent increases in cell numbers (data not shown) and DNA synthesis (Fig. 2), whereas 3αHP resulted in dose-dependent decreases, in each of the four breast cell lines. The effects of treating cell lines with 10^{-6} M of either 5αP, 3αHP, or 5αP + 3αHP are shown in Fig. 3. 5αP resulted in significant increases (generally $p < 0.001$) in cell numbers (Fig. 3A) and in [^{3}H]thymidine uptake (Fig. 3B) in each cell line. 3αHP resulted in significant suppression ($p < 0.05$ to $p < 0.001$) of cell proliferation (Fig. 3A) and [^{3}H]thymidine uptake (Fig. 3B) in each cell line. Treating the cells simultaneously with both 5αP and 3αHP resulted in partial or complete abrogation of the effect of either alone.

3.2. Effect of 5αP and 3αHP on mitosis

To determine if the observed changes in cell number were due to changes in the mitotic index, each cell line was treated with 10^{-6} M of either 5αP, 3αHP, or 5αP + 3αHP for 72 h and observed for nuclear morphology (Hoechst staining). The effects on mitosis were quantified by determining the number of mitotic figures as a percent of total number of cells and are shown in Fig. 4. 5αP resulted in significant increases ($p < 0.01$–0.001) in number of mitotic cells

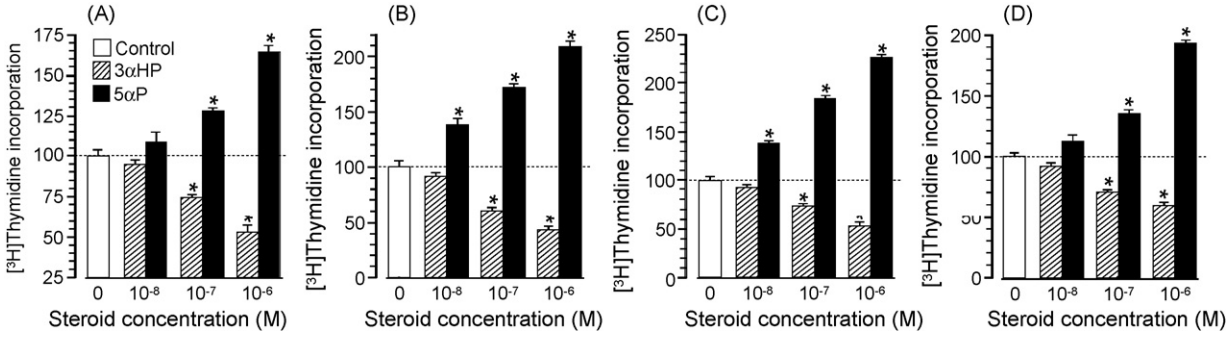

Fig. 2. Dose-dependent effects of 5αP and 3αHP on proliferation of human breast cell lines. Proliferation was determined in (A) MCF-10A, (B) MCF-7, (C) MDA-MB-231, and (D) T47D cells by [^{3}H]thymidine incorporation after 72 h of treatment at 0 (control), 10^{-8}, 10^{-7}, or 10^{-6} M of either 3αHP or 5αP. Results are presented as mean ± SEM, calculated as percent of control (open bars; 100%) from at least 3 separate experiments, each treatment having 4–6 replicates. *Significantly different from controls at $p < 0.001$.

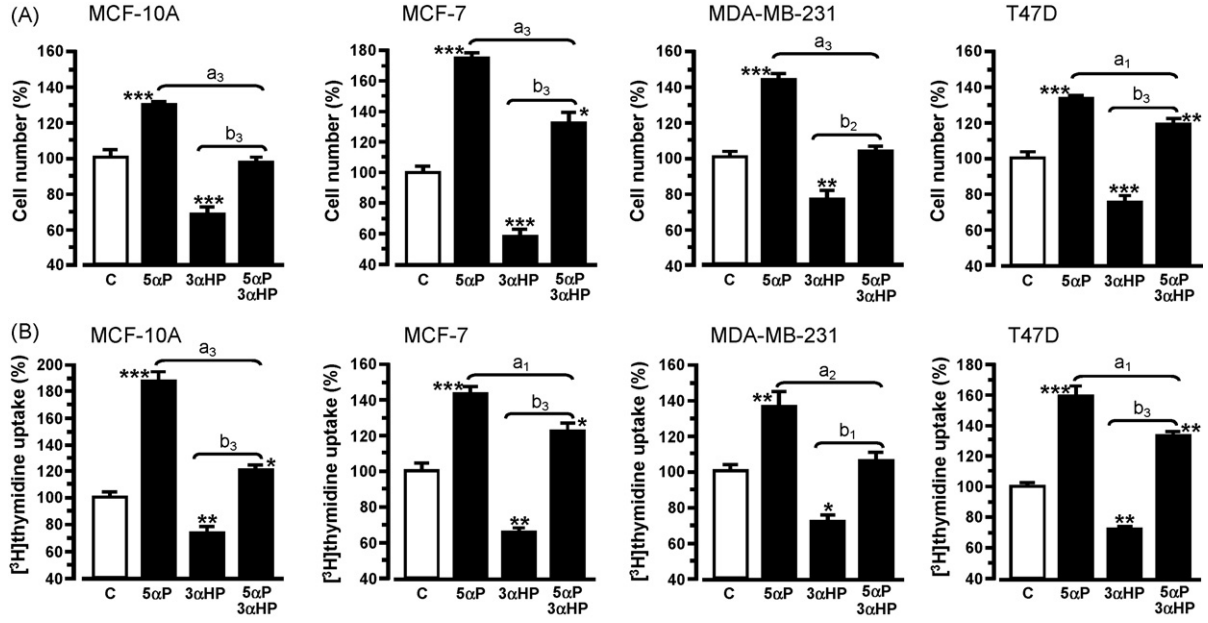

Fig. 3. Effects of 5αP, 3αHP and 5αP + 3αHP on proliferation of human breast cell lines. Cells (MCF-10A, MCF-7, MDA-MB-231 and T47D) were treated for 72 h without (C, control) or with 10^{-6} M 5αP and/or 3αHP, and proliferation was determined by (A) cell counts, and (B) [^{3}H]thymidine incorporation as described in Section 2. For each cell line, the results are presented as mean ± SEM, calculated as percent of control (100%) from at least 3 separate experiments, each treatment having 4–6 replicates. *, **, ***, significantly different from controls at $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively. a_1, a_2, a_3, significant differences at $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively, between 5αP and 5αP + 3αHP treatments. b_1, b_2, b_3, significant differences at $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively, between 3αHP and 5αP + 3αHP treatments.

in each of the cell lines. 3αHP did not significantly lower the mitotic rate below the control levels; however, 3αHP significantly ($p < 0.01$ to $p < 0.001$) suppressed the 5αP-induced increases in mitosis in each cell line.

3.3. Effect of 5αP and 3αHP on apoptosis

The effects of 5αP and 3αHP on apoptosis were quantified by three methods: (a) Hoechst staining to visualize cell change

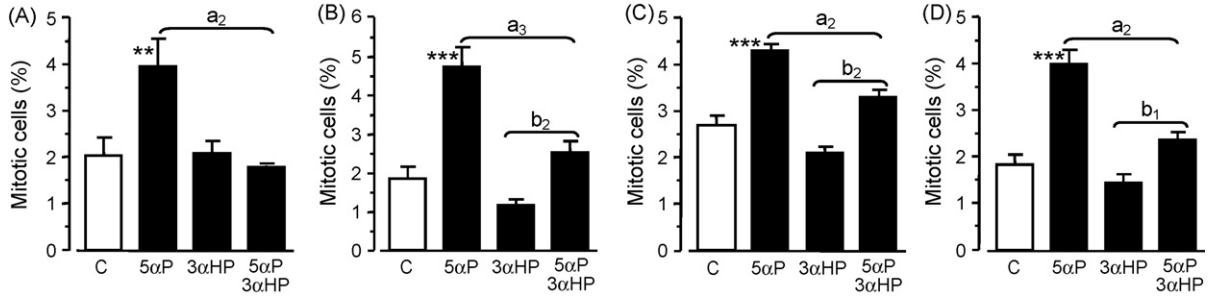

Fig. 4. Effects of 5αP, 3αHP and 5αP + 3αHP on mitosis in (A) MCF-10A, (B) MCF-7, (C) MDA-MB-231, and (D) T47D cells. Cells were treated for 72 h without (C, control) or with 10^{-6} M 5αP and/or 3αHP, stained with Hoechst and the number of mitotic nuclei scored as described in Section 2. For each cell line, the results are presented as mean ± SEM, calculated as percent of total number of cells examined per treatment from at least 3 separate experiments, each treatment having 4–6 replicates. **, ***, significantly different from controls at $p < 0.01$, $p < 0.001$, respectively. a_2, a_3, significant differences at $p < 0.01$ and $p < 0.001$, respectively, between 5αP and 5αP + 3αHP treatments. b_1, b_2, significant differences at $p < 0.05$ and $p < 0.01$, respectively, between 3αHP and 5αP + 3αHP treatments.

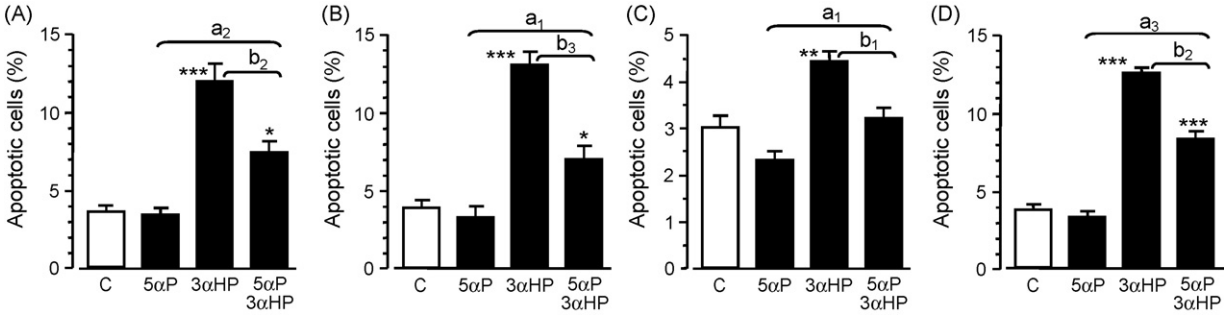

Fig. 5. Effects of 5αP, 3αHP and 5αP + 3αHP on apoptosis in (A) MCF-10A, (B) MCF-7, (C) MDA-MB-231, and (D) T47D cells. Cells were treated for 72 h without (C, control) or with 10^{-6} M 5αP and/or 3αHP, and processed and scored for apoptosis following Hoechst staining, annexin V staining or the TUNEL method as described in Section 2. For each cell line, the results are presented as mean ± SEM calculated as percent of total number of cells examined per treatment from at least 3 separate experiments, each treatment having 4–6 replicates. Because the results were essentially the same for all three methods, and to avoid redundancy, only the results for the Hoechst method are shown. *, **, ***, significantly different from controls at $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively. a_1, a_2, a_3, significant differences at $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively, between 5αP and 5αP + 3αHP treatments. b_1, b_2, b_3, significant differences at $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively, between 3αHP and 5αP + 3αHP treatments.

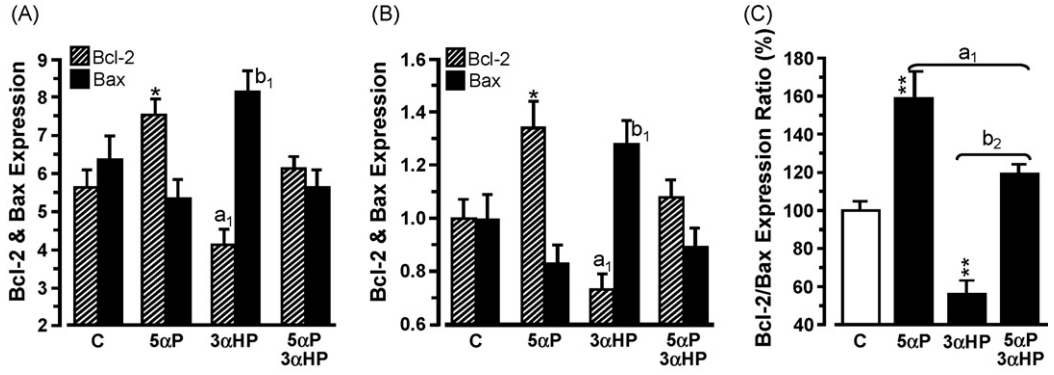

Fig. 6. Effect of 5αP, 3αHP and 5αP + 3αHP on Bcl-2 and Bax expression. MCF-7 cells were treated for 72 h without (C, control) or with 10^{-6} M 5αP and/or 3αHP as described in Section 2. Real-time PCR was conducted and expression levels were determined in relation to 18S rRNA. In (A) the results from a single experiment (with 4 replicates) are shown as Bcl-2 and Bax expression levels. In (B), the composite results from four separate experiments are shown as Bcl-2 and Bax expression levels standardized against the controls (set to a value of 1.0). In (C) the composite results from (B) are transformed to Bcl-2/Bax expression ratios and presented as percent of control (100%). Bars and lines show means and SEM. *, **, significantly different from controls at $p < 0.05$ and $p < 0.01$, respectively. (A) a_1, significantly different from 5αP Bcl-2 at $p < 0.001$, and significantly different from 5αP + 3αHP Bcl-2 at $p < 0.05$; b_1, significantly different from 5αP Bax, and from 5αP + 3αHP Bax at $p < 0.05$. (B) a_1, significantly different from 5αP Bcl-2 at $p < 0.001$, and significantly different from 5αP + 3αHP Bcl-2 at $p < 0.05$; b_1, significantly different from 5αP Bax at $p < 0.01$, and significantly different from 5αP + 3αHP Bax at $p < 0.05$. (C) a_1, significant differences at $p < 0.05$ between 5αP and 5αP + 3αHP treatments; b_2, significant differences at $p < 0.01$ between 3αHP and 5αP + 3αHP treatments.

and nuclear condensation as general parameters of apoptosis, (b) annexin V staining to detect changes in the plasma membrane occurring in the early stages of apoptosis, and (c) the TUNEL method as evidence of advanced stages of apoptosis. Because the results obtained by the three methods were essentially the same, and to avoid redundancy, Fig. 5 shows only the results from the Hoechst staining method. Although the range of apoptotic indexes varied between cell types, the overall results showed significant (2–4-fold; $p < 0.01$ to $p < 0.001$) increases in apoptosis due to 3αHP treatment. 5αP did not alter the incidence of apoptosis significantly from that of the controls in any of the cell lines examined and by none of the three methods; however, 5αP significantly reduced the 3αHP-induced increases in apoptosis of all four cell types.

3.4. Effect of 5αP and 3αHP on Bcl-2, Bax and p21 expression

MCF-7 cells were treated with 5αP, 3αHP or 5αP + 3αHP for 72 h and the effects on Bcl-2 and Bax expression were determined. Expression of Bcl-2 was increased with 5αP treatment (Fig. 6A and B), was insignificantly reduced ($p > 0.05$) by 3αHP and remained the same as in the controls in the presence of 5αP + 3αHP. Expression of Bcl-2 was significantly lower in cells treated with 3αHP than in cells treated with 5αP and in those treated with 5αP + 3αHP (Fig. 6A and B). The expression of Bax appeared to increase with 3αHP treatment but no statistically significant differences were noted due to the treatments; however, expression of Bax was significantly higher in cells treated with 3αHP than in cells treated with 5αP and in those treated with 5αP + 3αHP (Fig. 6A and B). When expression levels of Bcl-2 and Bax were considered as ratios of Bcl-2/Bax, and

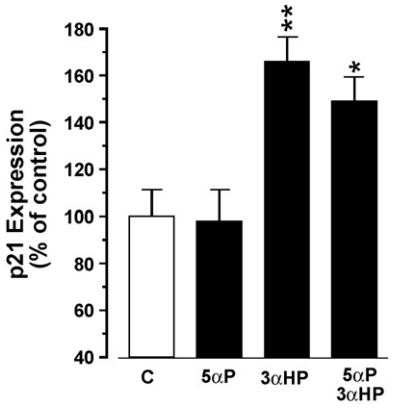

Fig. 7. Effect of 5αP, 3αHP and 5αP + 3αHP on p21 expression. MCF-7 cells were treated for 72 h without (C, control) or with 10^{-6} M 5αP and/or 3αHP as described in Section 2. Real-time PCR was conducted and expression levels were determined in relation to 18S rRNA. Bars and lines show means ± SEM of p21 expression levels from four separate experiments, presented as percent of control (100%). *, **, significantly different from controls at $p < 0.05$ and $p < 0.01$, respectively.

the combined results from four separate experiments were calculated as percent of the controls (Fig. 6C), there were significant increases ($p < 0.01$) with 5αP and significant decreases ($p < 0.01$) with 3αHP treatments. Concomitant treatment with both 5αP and 3αHP resulted in significant ($p < 0.05$) reduction of the 5αP-induced increase in the Bcl-2/Bax expression ratio and in significant ($p < 0.01$) reversal of the 3αHP-induced suppression (Fig. 6C).

The effects of 5αP and 3αHP on the expression of p21, a cell cycle inhibitor, are shown in Fig. 7. Treatment with 3αHP resulted in significant ($p < 0.01$) increases, whereas 5αP had no significant effect, on p21 expression.

4. Discussion

4.1. Effects of 5αP and 3αHP on cell replication

Net increases in cell numbers result from either an increase in the rate of cell proliferation or a decrease in cell death, or both [18]. It follows that stimulation (growth) or regression of a tumor can result from deregulation of either or both of these processes. Knowledge of hormonal influences on proliferation and apoptosis are therefore of significance in understanding and treating hormone-sensitive cancers such as breast cancer. Previous studies [1,2,8] had shown that the progesterone metabolite, 5αP, stimulates, whereas 3αHP suppresses, proliferation of human breast cells. In those studies the overall effect on cell numbers by individual treatment of either 5αP or 3αHP were examined. Subsequent studies in which progesterone conversion to 5αP was blocked by the 5α-reductase inhibitor, dutasteride, provided proof-of-principle that stimulation of cell proliferation is not due to progesterone but to the 5α-reduced metabolite 5αP [19]. The current proliferation studies confirmed by two independent methods (cell counts and [^{3}H]thymidine uptake) that 5αP and 3αHP have opposing effects and that the effects are observed in each of the four breast cell lines examined, regardless of ER/PR status and tumorigenicity potential. The results showed that 5αP increases, and 3αHP decreases, proliferation of each cell line, confirming earlier findings [1,2,8]. In addition, we now report that the effect of either 5αP or 3αHP is significantly reversed when 5αP and 3αHP are administered together. Thus the 5αP-induced increases in cell number and DNA synthesis are suppressed by 3αHP and, similarly, the 3αHP-induced decreases are significantly countered by 5αP.

4.2. Effects of 5αP and 3αHP on mitosis

Growth curves do not assess the relative contributions of mitotic and apoptotic rates. The effects of 5αP and 3αHP on cell numbers observed following 72 h of treatment could be due to different effects of either hormone on mitosis and/or apoptosis, or both, and could be different for different cell types. In the current studies, we examined the relative effects of 5αP and 3αHP on mitosis in the four breast cell lines by quantifying the number of Hoechst stained cells undergoing mitosis. The studies show that 5αP resulted in significant increases in the mitotic rate in each of the four cell lines. On the other hand, 3αHP did not significantly decrease mitotic rate below the control level, when measured by the microscopic method. However, 3αHP significantly suppressed the 5αP-induced increases in mitotic rate. From the results it would appear that at 10^{-6} M, mitotic stimulation by 5αP is greater than mitotic inhibition by 3αHP. The fact that significant reductions in mitotic indices below control levels by treatment with 3αHP alone were not registered could be due to the lack of sensitivity of the microscopic method employed. The number of mitotic figures in untreated cells was low (around 2%) and a significant reduction below this level might only be observed if many more fields of cells were analyzed. Incorporation of [^{3}H]thymidine is a sensitive and objective method and the results obtained by this method clearly showed that 3αHP resulted in significant reduction, in relation to controls, in DNA synthesis, an antecedent of mitosis.

4.3. Effects of 5αP and 3αHP on apoptosis

Increases in cell numbers *in vitro*, and tumor growth *in vivo*, depend on the suppression of apoptosis as well as stimulation of cell proliferation. To determine the effects of 5αP and 3αHP on apoptosis, three different methods that detect morphological and biochemical changes known to be associated with apoptosis at early and late stages were employed. By all three methods it was evident that 3αHP caused significant increases in number of cells undergoing apoptosis, and the increases occurred in each of the four cell lines. In turn, 5αP significantly suppressed 3αHP-induced apoptosis but did not result in apparent significant changes in percent of cells undergoing apoptosis in comparison with the controls. The fact that significant reductions in apoptosis, below control levels, by 5αP treatments were not registered, could be due to the lack of sensitivity of the microscopic methods employed, especially at the lower apoptotic frequencies, or it could signify that the primary action of 5αP with respect to cell proliferation is not on apoptosis. Overall, the results show that 3αHP significantly increases apoptosis and 5αP can suppress some or all of these increases.

Other studies have shown that apoptosis is influenced by steroid hormones such as estradiol and progesterone. Estradiol resulted in suppression of apoptosis in MCF-7, T47D, or ZR75-1 human breast cancer cell lines [20–24] and in breast tissue explants [25]. In this regard, estradiol appears to act similarly to 5αP. Anti-estrogens, such as tamoxifen, have been shown to induce apoptosis in breast cancer cells [21,26]. The changes in level of apoptosis in the mammary gland during the menstrual cycle also have been linked to changes in estradiol levels [27]. Progesterone at high levels (10^{-5} to 10^{-4} M) has been shown to increase apoptosis in MCF-7 and T47D cells [28,29] and in malignant mesothelioma cells [30], and incubations with progesterone at 10^{-7} M resulted in reduction of serum depletion-induced apoptosis in MCF-7 and MDA-MB-231 cells [31,32]. These progesterone studies need to be interpreted in the light of rapid progesterone transformations to 5α-pregnanes, such as 5αP, and 4-pregnenes such as 3αHP by breast cell lines. For example, we have shown previously [3,19] that MCF-7, MDA-MB-231 and T47D cells convert 80–90% of added progesterone into 5α-pregnanes and 4-pregnenes in 8–24 h, and up to 99% (MCF-7 cells) in 72 h [19]. In each of these cell lines, the 5α-pregnanes constitute 80–86% of all progesterone metabolites after an 8 h incubation [3], and >90% after 24 h and >97% after 48 h [19]. On the other hand, progesterone conversion by non-tumorigenic MCF-10A cells results in substantially lower 5α-pregnane:4-pregnene ratios in total [3], and specifically 10–30 fold lower 5αP:3αHP ratios [19], than by tumorigenic MCF-7 cells. These findings suggest that the results of reported studies of the effects of progesterone on cell proliferation and/or apoptosis should be interpreted in the light of the progesterone metabolites formed rather than the progesterone that was added.

4.4. Effects of 5αP and 3αHP on Bcl-2 and Bax expression

A major component of apoptotic pathways involves caspases which, when activated, can result in the digestion of structural proteins in the cytoplasm, degradation of chromosomal DNA and general dysregulation of cellular functions [33,34]. Activation of the caspase cascade occurs when cytochrome *c* is released from the inter-membrane space of mitochondria into the cytosol [34,35]. The release of cytochrome *c* is regulated by anti- and pro-apoptotic

proteins, the majority of which come from the Bcl-2 family [36]. Bcl-2 has been termed anti-apoptotic because it can act to inhibit the release of cytochrome *c* [35], whereas the Bcl-2-associated X protein, Bax, promotes the release of cytochrome *c* and therefore is considered to be pro-apoptotic [36]. A number of studies have noted the importance, not of absolute levels of Bcl-2 and Bax, but rather of the ratio of the two: a high Bcl-2/Bax ratio is associated with decreased apoptosis and a low ratio with increased apoptosis [33,35,37,38]. In addition, Bcl-2 is high in tumor and low in normal tissue, whereas Bax is low in tumor and high in normal tissue [39].

Our studies reported here indicate that the regulation of apoptosis by 5αP and 3αHP involves actions at the level of Bcl-2 and Bax expression. 5αP, which inhibited apoptosis, resulted in significant increases in Bcl-2/Bax expression ratios. 3αHP, which significantly increased apoptosis, resulted in significantly decreased Bcl-2/Bax expression ratios. The expression results were consistent with the apoptosis and proliferation data. The opposing actions of 5αP and 3αHP on this pathway were further demonstrated by the reversal of effects of each when cells were treated simultaneously with both. The pro-apoptotic effects of 3αHP and the concomitant down-regulation of Bcl-2/Bax ratios are similar to the actions described for anti-estrogens [40,41], the 17β-estradiol metabolite 2-methoxyestradiol [42] and progestins [22,28,40] on breast cell lines. The anti-apoptotic and pro-proliferation effects of 5αP and the upregulation of Bcl-2/Bax ratios are similar to the reported actions of estradiol on breast cell lines such as MCF-7, T47D; ZR-75-1 [22–24,43].

4.5. Effects of 5αP and 3αHP on p21 expression

The protein, p21, is a member of the family of cyclin kinase inhibitors [17,44] which contain a conserved region of sequence at the N-terminus that can bind and inhibit cyclin dependent kinase (Cdk) complexes [16]. Overexpression of p21 leads to G_1, G_2 [45] and S-phase [46] arrest, thus suppressing cell proliferation. p21 transcription is directly regulated by the tumor suppressor gene p53 in response to DNA damage or other cellular perturbations [47,48] and therefore increased expression of p21 has been considered as a marker for apoptosis induction [49]. In our studies, 3αHP induced significant increases in p21 expression, which were consistent with the observed decreases in mitosis and cell proliferation and the increases in apoptosis, as well as the associated decreases in Bcl-2/Bax ratios. Similar changes were observed upon estrogen withdrawal in MCF-7 tumors which led to induction of apoptosis and inhibition of proliferation due to an increase in p21 and a decrease in Bcl-2 [20,22,24,38,39]. The lack of significant changes in p21 expression due to 5αP suggest that 5αP may not play a significant role in the deregulation of cell cycle arrest, at least not in terms of the cyclin kinase inhibitor, p21.

4.6. Mechanisms of action leading to the opposing effects of 5αP and 3αHP

The cellular signaling mechanisms of action leading to the opposing effects of 5αP and 3αHP are currently under investigation. Since specific receptors for 3αHP and 5αP have been demonstrated on the plasma membrane of MCF-7 and MCF-10A cells [6,8], the suggestion is that these steroid hormones act via membrane receptor-linked cell signaling pathways, in particular the mitogen-activated protein kinase (MAPK) and the protein kinase C (PKC) cascades. The MAPK/extracellular signal-regulated kinase (MAPK/Erk) signaling pathway is known to play a central role in the regulation of cell proliferation, survival, and apoptosis [50]. Activation of the MAPK/Erk pathway stimulates cell proliferation and suppresses apoptosis [51–53], and blocking the pathway is being considered for the treatment of cancer [54]. Our previous studies with MCF-7 cells [7] showed that 5αP treatment resulted in significant, dose-dependent increases in activated Erk1/2 while the MEK inhibitor, PD98059, resulted in significant suppression of the 5αP-induced MAPK activation. Similarly, 5αP-induced increases in replication and detachment were abrogated by PD98059 [8]. Those findings suggest that 5αP actions may involve stimulation of the MAKP/Erk cascade. The PKC cell signaling cascade also is known to be involved in the regulation of apoptosis, proliferation, growth factor response, and tumor promotion [55–57]. Thus activation of PKC by phorbol esters prevents apoptosis in response to growth factor withdrawal, while pharmacologic inhibition of PKC stimulates apoptosis and down-regulates Bcl-2 expression. Although the interaction of 3αHP with the PKC pathway in breast cells has not been examined, our studies on pituitary cells showed that 3αHP suppresses PKC action and the mobilization of intracellular Ca^{2+} [58,59]. We suggest that the observed pro-apoptotic and anti-proliferative actions of 3αHP could likewise be due to interaction with the PKC cell signaling cascade. Finally, since MAPK and PKC pathways interact, it is possible that 5αP and/or 3αHP have opposite actions on either or both of these pathways and these possibilities await investigation.

4.7. Summary

In summary, the current investigations have shown that 5αP promotes and 3αHP inhibits cell proliferation by differentially affecting mitosis and apoptosis. The overall increase in cell numbers resulting from 5αP treatment is due primarily to increased stimulation in mitosis and secondarily to suppression of apoptosis involving increases in Bcl-2/Bax ratios. On the other hand, 3αHP treatment decreases cell proliferation by both decreasing replication and increasing apoptosis; 3αHP increases apoptosis at the genetic level as evidenced by a decreased Bcl-2/Bax ratio, as well as an increase in the p53 activated cell arrest protein p21. Combination treatment of 5αP and 3αHP at equimolar levels abrogates the effects of either hormone on the processes studied. Importantly for breast cancer is that the opposing effects of 5αP and 3αHP are seen in all breast cell lines examined and the data suggest that all breast cancers (estrogen-responsive and unresponsive) might be suppressed by blocking 5αP formation and/or increasing 3αHP. The findings further support the hypothesis that progesterone metabolites are key regulatory hormones and that changes in their relative concentrations in the breast microenvironment determine whether breast tissues remain normal or become cancerous.

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